UNIVERSITY OF COPENHAGEN

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FACULTY OF LIFE SCIENCES
UNIVERSITY OF COPENHAGEN

Overview of pathogenesis, epidemiology and diagnostic tools necessary for successful surveillance and eradication of *Salmonella* Dublin from the Danish cattle population

Prize assignment
"Professor Dr.med.h.c.
C.O. Jensens Mindefond"

by

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Preface

This report was written as a prize assignment to Professor Dr.med.h.c. C. O. Jensen's Memorial Foundation ("Professor Dr.med.h.c. C. O. Jensens Mindefond"). The call for prize assignments was made public in relation to the 100-year celebration of the establishment of the National Veterinary Laboratory (Statens Veterinære Serumlaboratorium) in 1908.

The assignment description from the foundation was a description of an infection of importance in Danish production animals with focus on one or more of the following elements: Development of diagnostics, pathogenesis, immunity, treatment and epidemiology with perspectives for improvement of control and prevention of the infection in Denmark. The report had to contain results of the author's own research.

This report concerns *Salmonella* Dublin which is a bacterial infection of importance for the cattle population and for food safety. The report covers all subjects of relevance for control and eradication of *Salmonella* Dublin. It includes a review of state of the art pathogenesis, diagnostic tests and test strategies on animal and herd level, epidemiology including infection dynamics within and between herds, intervention and surveillance of *Salmonella* Dublin in Denmark based on available literature and results of own research.

The subject is historically interesting because professor Dr.med.h.c. C.O. Jensen was the first one to describe *Salmonella* Dublin outbreaks and bacteria characteristics in Denmark. He also reproduced the disease by experimental infections back in 1891 and to my knowledge these are the first written reports that concern this infection. This report therefore reviews a lot of the relevant research from recognition to control of the infection.

I would like to thank Professor Hans Houe (Faculty of Life Sciences, University of Copenhagen), Principal Research Scientist David Jordan (Department of Primary Industries, NSW, Australia) and Chief Scientist Lis Alban (Danish Meat Association) for constructive criticism of this report. I would also like to take the opportunity to thank the Danish Cattle Federation, Danish Dairy Board, The Milk Levy Fund, The Cattle Levy Fund and The Danish Food Industry Agency for funding and supporting the research I was involved in from 2000-2009 in the field of *Salmonella* Dublin epidemiology, which allowed me to gather the knowledge presented in this report.

Liza Rosenbaum Nielsen, DVM, PhD, Dipl.ECVPH
Frederiksberg, 20th of February, 2009

Summary in English

Salmonella enterica subsp. *enterica* serovar Dublin (*S.* Dublin) is a cattle-adapted type of bacteria which can cause severe illness in animals and humans. *S.* Dublin is common in Danish cattle. About 14 % of dairy herds and up to 38 % of dairy-beef herds are presently showing evidence of current or recent infection. The resulting impact on the welfare of animals, production loss and public health has made *S.* Dublin a top priority with the Danish cattle industry and the Danish Veterinary and Food Administration. Consequently, a surveillance program was initiated in 2002, which has dramatically reduced the number of infected cattle. From 2007 an eradication campaign was initiated with the aim of eradicating *S.* Dublin from the Danish cattle population by the end of 2014. This report describes the research behind this success and demonstrates that progress on such a large scale requires the integration of efforts of many different scientific disciplines. While knowledge of disease development (pathogenesis), validity and interpretation of diagnostic tests, epidemiology, intervention and surveillance are all essential to control *S.* Dublin in cattle, only the *combination* of these constituent efforts into a coherent plan may lead to solution of such a complex and persistent problem.

A literature review reveals that most knowledge about mechanisms of *S.* Dublin pathogenesis comes from experimental studies. Unfortunately, species used in those studies are rarely relevant to extrapolate conclusions to cattle. Further, experimental pathogenesis studies often provide conclusions about small – sometimes contradictory - pieces in the puzzle, but never provide a more unifying interpretation. So we are left with just a few consistent pieces of information useful for understanding how to control and eventually eradicate the infection in practice: i) cell-mediated immunity is more important for protection of cattle against *S.* Dublin infection compared to humoral immunity (antibody production), ii) antibodies may be useful for diagnostics, because they provide evidence of current or recent infection, but they do not provide sufficient immune protection against this highly invasive infection. However, methods to improve cell-mediated immunity, such as vaccinations, are unlikely to be profitable and sufficient for eradication, because studies show that they only provide partial protection and do not completely stop excretion of bacteria from infected cattle. Although the pathogenesis of *S.* Dublin at molecular level is intriguing and academically interesting, it is unlikely that our lack of understanding of the details at this level is an important hindrance of successful intervention in cattle herds today.

Multi-institutional research collaboration during 2000 to 2009 in Denmark has added a lot of new and useful knowledge about disease development. We have established duration and amount of bacteria shed in faeces from infected cattle under field conditions, suggested risk profiles for individual animals based on repeated testing, determined risk factors for carrier development and challenged previously suggested methods for carrier detection, which has modified the recommendations in practice. Further, we have studied how diagnostic test results can be interpreted to improve performance of the currently available tests when used for specific purposes as illustrated in this report.

Currently no individual, highly sensitive bacteria-detection test exists. Such tests would be useful for rapid and accurate outbreak diagnosis towards the end of the eradication campaign. On the other hand, the currently available diagnostic tests for antibody measurements in bulk tank milk and individual samples (ELISA) can be combined to obtain good sensitivity already a few weeks after the start of an outbreak of *S.* Dublin. Real-time Polymerase Chain Reaction (PCR) methods may be a solution to improving the sensitivity of bacterial detection when serotyping is not essential. We are currently collecting field samples which will be used to establish the validity of a real-time PCR-test being developed at the National Food Institute.

The wide variations in the infection course and clinical expression of *S.* Dublin infections in cattle herds make it challenging to communicate advice to farmers about how to control the infection. Many advisers and farmers seek simple, straightforward recommendations similar to those used in previous eradication campaigns such as the successful BVD eradication campaign, where test-and-cull-procedures played a central role. For *S.* Dublin, simple recommendations from the experimental studies about culling of carrier animals detected by repeated antibody measurements need to be used with care. Due to faecal excretion and survival outside the host *S.* Dublin has a strong environmental component that needs to be taken into account. Based on our research and experiences, we only recommend culling to avoid re-infections if other intervention actions are already evidently successful in reducing spread of *S.* Dublin among young stock.

On-going collaborative research is aimed at developing a detailed simulation model to study herd specific intervention scenarios in which the current knowledge about disease development, infection dynamics, test-interpretation and production effects at age group and herd level can be incorporated. If such a simulation tool could become available for local advisers it could provide an important motivation for the farmer, because the advice given could be made herd specific and add economical consequences to the argumentation while taking into account herd size, management and culling strategies in the herd.

Motivation of the farmers is essential to obtain a successful eradication campaign. Experiences from our *S.* Dublin field intervention trials and previous eradication campaigns have shown that a wide range of activities and tools are necessary to be successful on the large scale. Communication is essential. It can be divided into three subgroups:

1. Local direct communication: Small experience groups and continuous follow-up by local advisers. This requires that the local advisers are motivated to work with the problem. Local politicians may be able to advance that process.
2. Written material: Broad, repeated and long-term communication via farmer's magazines, meetings, farmer congresses, folders etc. This can be organised centrally by farmer's organisations
3. Participation in research projects: It should not be underestimated that farmer' and advisers' direct participation in projects improve the understanding of the infection and commitment to intervene. Further, examples from the Kongeå-project in Southern Jutland in 1999-2003 showed that such projects can help demystifying taboo-hampered infections.

Also, farmers need access to tools that can help them protect their herd against *S.* Dublin. This is to some extend provided by publicly available herd infection status on the internet. We have also developed a manual for risk assessment and planning of intervention. Moreover, planned price differentiations from 2010 will undoubtedly motivate more farmers to start intervention. It will complicate selling cattle from herds with poor infection status because farmers will become more demanding about the important external biosecurity.

I believe that the research community could provide the public, politicians and the cattle industry with estimates of the socioeconomic benefits of the *S.* Dublin-surveillance and eradication campaign. Already millions of DKK have been spent on the research and development to reach the point we are at today. Another 100 million DKK will be spent over the next 5 years on projects, intervention and surveillance. Even though it is difficult to put a value to the loss of human beings, the currently estimated one to four Danish beef-associated *S.* Dublin-case fatalities per year calls for an effort to weigh cost-benefit both at herd level, sector level and for public health. It would also be highly beneficial to know more about the human *S.* Dublin-cases to examine, if some of these cases could or should be avoided through improved biosecurity elsewhere in the farm-to-fork-chain than in the primary production. Finally, we need to find out which import restrictions would help us reduce the number of human *S.* Dublin-cases and human *S.* Dublin-case fatalities from imported meat once after the infection is eradicated from Danish cattle.

Sammendrag på dansk (Summary in Danish)

Salmonella enterica subsp. *enterica* serovar Dublin (*S.* Dublin) er en kvægtilpasset bakterie, der kan give alvorlig sygdom hos dyr og mennesker. Der er tegn på infektionen i 14 % af danske malkekvægsbesætninger og op mod 38 % af slagtekalvebesætninger. Konsekvenserne af infektionen er nedsat dyresundhed og produktionstab i besætningerne samt smitterisiko for mennesker i kontakt med smittede dyr samt forbrugere af oksekødsprodukter. Derfor er *S.* Dublin prioriteret højt på dagsordenen i kvægbruget og Fødevarestyrelsen, som sammen har startet et overvågningsprogram i 2002. Dette har reduceret forekomsten i kvæg betydeligt, og indsatsen fortsættes i en saneringskampagne, der har til formål at udrydde infektionen fra kvægbruget inden udgangen af 2014. Denne rapport beskriver den forskning og udvikling, som har gjort den hidtidige succes mulig. Den demonstrerer endvidere, at for at nå målet kræves indsats på tværs af mange videnskabelige discipliner. Viden om sygdomsudvikling (patogenese), diagnostisk test validitet, fortolkning af testresultater, epidemiologi, intervention og overvågning er hver især nødvendige for succesrig bekæmpelse af *S.* Dublin i kvæg. Det er dog kun ved at *kombinere* alle disse vigtige komponenter i en samlet plan at sådan et komplekst og persisterende problem kan håndteres.

Litteratursøgning viser, at den meste af den viden vi har i dag om *S.* Dublins patogenesemekanismer kommer fra eksperimentelle studier. Desværre er de dyrearter, der bliver brugt i disse studier ikke altid lige relevante for kvæg. Ydermere giver de eksperimentelle patogenesestudier oftest svar på mindre, nogle gange modstridende, brikker i det store puslespil uden at give en mere samlet forståelse for mekanismerne og sygdomsudviklingen. Således står vi i dag i en situation, hvor vi på trods af stor indsats indenfor patogeneseforskningen, kun har meget få væsentlige og konsistente konklusioner, som er relevante for bekæmpelse af *S.* Dublin i praksis; i) cellemedieret immunitet er tilsyneladende vigtigst for beskyttelse og bekæmpelse af *S.* Dublin i kvægets immunsystem sammenlignet med antistofdannelse og ii) antistofferne kan være nyttige til diagnostiske formål, fordi de oftest påviser nylig infektion, men de giver ingen information om dyrets evne til at modstå og bekæmpe denne meget invaderende infektion. Metoder til at forbedre det cellemedierede immunforsvar, så som vacciner, er dog ikke udbytterige og tilstrækkelige i forbindelse med en saneringskampagne, fordi de kun yder delvis beskyttelse og ikke stopper udskillelsen af *Salmonella*-bakterier helt. Selvom mysteriet om *S.* Dublins patogenese på celleniveau er både akademisk og grundforskningsmæssigt spændende, er det ikke sandsynligt at vores mangel på forståelse af de cellulære mekanismer står i vejen for en succesrig saneringskampagne i kvægbruget i dag.

Forskningssamarbejde mellem flere institutioner i Danmark fra 2000 til 2009 har tilføjet megen ny og nyttig viden om sygdomsudvikling. Vi har bestemt varigheden af udskillelse og koncentrationer i fæces fra *S*. Dublin smittede dyr under naturlige forhold. Vi har udviklet risikoprofiler for kvæg baseret på gentagne målinger, fundet risikofaktorer for udvikling af smittebærerstadiet og justeret tidligere foreslåede metoder til udpegning af persisterende smittebærer. Derudover har vi fundet frem til hvordan diagnostiske testresultater kan fortolkes for at forbedre performance af de forhåndenværende tests, når de bruges til specifikke formål.

Der findes i dag ingen diagnostiske tests med høj følsomhed for fund af *S.* Dublin-bakterier i kvægprøver. Sådanne tests ville være nyttige til hurtig og korrekt diagnosticering af udbrud især i slutningen af en saneringskampagne. Det er dog muligt at kombinere eksisterende antistoftest (ELISA) til tankmælksprøver og individprøver, så der opnås en høj følsomhed allerede et par uger efter et nyudbrud, og metoden kan også bruges til fritestning af sanerede besætninger. Real-time Polymerase Chain Reaction (PCR) metoder kan formentlig forbedre følsomheden af dyrkningstests i situationer, hvor det ikke er vigtigt at kende serotypen. I et igangværende projekt indsamler vi prøver fra besætninger for at evaluere validiteten af en real-time PCR-test, som er under udvikling på Fødevareinstituttet.

De store variationer i infektionsforløbet og kliniske tegn på *S*. Dublin i kvægbesætninger gør det vanskeligt at kommunikere anbefalinger til landmænd om bekæmpelsesmetoder. Mange rådgivere og landmænd søger enkle anbefalinger i stil med dem der har været brugt i tidligere saneringskampagner, som fx den succesfulde udryddelse af Bovin Virus Diarré (BVD), hvor test- og udsætningsstrategier var helt centrale i bekæmpelsen. Men test- og udsætning af persisterende smittebærere af *S.* Dublin baseret på gentagne antistofmålinger skal bruges med omtanke. Denne bakterie udskilles med fæces og har et væsentligt større smittereservoir i miljøet omkring dyrene end BVD, og testmetoden til udpegning giver mange falsk positive. Baseret på vores erfaringer anbefaler vi i dag først udsætning af potentielle smittebærere, når andre interventionstiltag er påviseligt effektive til at reducere smittespredningen blandt ungdyrene i besætningen.

I et igangværende samarbejdsprojekt forsøger vi at udvikle en detaljeret simuleringsmodel til at studere besætningsspecifikke interventionsscenarier. I modellen integreres vores viden om sygdomsudvikling, infektionsdynamik, testfortolkning og produktionseffekter på aldersgruppe- og besætningsniveau og bestemte scenarier afprøves til publikation og generel information. Hvis sådan et simuleringsværktøj kunne gøres tilgængeligt for lokale rådgivere, kunne det give ekstra motivation for landmændene, fordi de råd der så gives kan gøres besætningsspecifikke og inkludere økonomiske konsekvenser samtidig med at der tages højde for besætningsstørrelse, management og udsætningsstrategier i besætningen.

Netop landmændenes motivation er essentiel for at nå målet i saneringskampagnen. Erfaringer fra vores S. Dublin interventionsstudier og tidligere kampagner har vist at en bred vifte af aktiviteter og værktøjer er nødvendige. Kommunikation er meget vigtig og kan inddeles i tre undergrupperinger:

1. Lokal og direkte kommunikation: Små erfaringsudvekslingsgrupper/staldskoler og løbende opfølgning fra rådgivernes side. Dette kræver at rådgiverne er motiverede for at arbejde med problemet. Lokale politikere i kvægbruget kan være med til at stimulere indsatsen.
2. Skriftligt materiale: Gentagne gange informeres om faglige emner og beslutninger i saneringskampagnen gennem fagblade, møder, landmandskonferencer, pjecer osv. Dette kan organiseres centralt fra af kvægbrugets egne organisationer.
3. Deltagelse i projekter: Man bør ikke undervurdere den effekt det har på landmænds og rådgiveres forståelse for infektionen og motivation til at sanere, hvis de inviteres til at deltage i projekter. Eksempler fra Kongeåprojektet i Sønderjylland i 1999-2003 viste endvidere at sådanne projekter kan være med til at afmystificere tabu-behæftede emner og infektioner.

Landmændene har brug for adgang til værktøjer som kan hjælpe dem med at beskytte deres besætning mod *S.* Dublin. Dette er til en vis grad muligt via de offentligt tilgængelige *Salmonella*-overvågningsniveauer på internettet. Desuden har vi udviklet en manual til risikovurdering i stalden og planlægning af besætningsspecifikke handlingsplaner. De planlagte prisdifferentieringer på kød og mælk fra 2010 vil utvivlsomt motivere flere landmænd til at gå i gang med at sanere. Det vil blive svært at sælge dyr fra besætninger med ringe infektionsstatus, fordi kravene til ekstern smittebeskyttelse øges i de enkelte besætninger.

Jeg mener, at forskning kan afdække de socioøkonomiske konsekvenser ved *S*. Dublin overvågnings- og saneringskampagnen for offentligheden, politikerne og kvægbranchen. Der er allerede brugt millioner af kroner på forskning og udvikling for at nå hertil, og yderligere ca. 100 mill. kr. vil blive brugt over de næste 5 år på forskning, sanering og overvågning i Danmark. Selvom det er vanskeligt at værdisætte et menneskeliv, så bør der med et til fire årlige *S*. Dublin-forårsagede dødsfald hos mennesker relateret til dansk oksekød kunne gøres en indsats for at vurdere cost-benefit af programmerne både på besætnings- og sektorniveau samt for befolkningen. Det ville også være meget nyttigt at undersøge risikofaktorer hos human-tilfælde af *S*. Dublin så det kan vurderes om øget hygiejne et andet sted i kæden end primærproduktionen kunne forhindre nogle af de humane tilfælde. Slutteligt bør vi undersøge, hvilke importrestriktioner, der kan hjælpe os med at reducere antallet af S. Dublin-tilfælde hos mennesker stammende fra importeret kød.

1. Introduction

1.1 Background

Salmonella enterica subsp. *enterica* serovar Dublin (*S.* Dublin) is an infection that receives much attention in the Danish cattle industry and the Danish Veterinary and Food Administration. There are several reasons for this:

1. The infection is widespread and common in some parts of the Danish cattle population with an average seroprevalence of 14.1% amongst dairy herds in January 2009 (www.kvaegvet.dk). Moreover, 38% of slaughter calf production sites that deliver more than 100 animals to slaughter every year are seropositive.

2. The infection compromises animal health and welfare in infected herds and leads to economic losses in herds with active spread of infection (Richardson and Watson, 1971; Wray and Snoyenbos, 1985; Peters, 1985; Visser et al., 1997).

3. It is a zoonosis that can lead to severe, invasive infections in humans (Schønheyder et al., 1997; Helms et al., 2003) most likely after consumption of contaminated beef, intake of unpasteurized milk products (Maguire et al., 1992) or direct contact to infected cattle (Mateus et al., 2008).

Facts about animal health and economic consequences of *S.* Dublin in cattle herds

A field study of 223 infected herds suggested that *S.* Dublin leads to adult dysentery in 18 %, abortions in 13 % and calf hood disease such as diarrhoea and pneumonia in 86 % of herd outbreaks. The associated mortality was 47 % out of 60 individual cows with dysentery whereas none of the aborting cows died. In total, 33 % of calves (n=6239) in these herds became clinically ill and half of the diseased calves died. However, there were large variations between herds (Richardson and Watson, 1971).

A study about economic consequences of *S.* Dublin found the cost of an outbreak in a 214 head dairy-beef calf rearing unit to be £4691 or £25.36 per survivor in 1982-numbers. This was concluded to be a substantial proportion of the gross margin of animals sent to slaughter after the outbreak (Peters, 1985). The main causes for the losses were increased calf mortality and veterinary expenses.

Another study quantified losses ascribed to *S.* Dublin in 40 dairy farms in the Netherlands. They found that losses related to abortions were most important. This is due to other effects related to the abortion such as culling patterns, loss of milk production and prolonged calving intervals (Visser et al., 1997). Calf mortality and veterinary expenses were also important losses. The average total loss was 5000 Dutch guilders (or 55 guilders per cow), but could go up to 18.000 Dutch guilders in the worst cases (1997-numbers). This was approximately 4.5 % of the net return to labour and management.

In current Danish numbers the above corresponds to approximately 50.000 DKK losses per year in a 150 “average” cow dairy herd, or 675 DKK per produced slaughter calf in fattening dairy-beef herds.

It should be noted that production and animal health losses caused by *S.* Dublin are herd-specific. Due to the complexity of the effects they should preferably be estimated for each herd i.e. by simulation modelling.

Facts about *S.* Dublin in humans

Even though the recorded number of human cases per year in Denmark is low, it is still considered unacceptable by the Ministry for Food, Agriculture and Fisheries (Anonymous, 2006). This is also due to a high case fatality rate for this particular serotype of *Salmonella*.

A register study of human gastroenteritis cases in Denmark from 1991 to 1999 found that the relative adjusted mortality of *S.* Dublin was 12.35 (95%CI: 6.67 to 22.86). For comparison, the relative adjusted mortality for *S.* Typhimurium was 2.88 (95%CI: 2.42 to 3.44). The interpretation is that people who experience salmonellosis from *S.* Dublin are more than 12 times more likely to die within one year after the incidence than other people of their own age, sex and country.

In cases with no other known or underlying diseases, the probability of dying was 17.6% which is high compared to other common gastroinstestinal infections (Helms et al., 2003). We have had between 13 and 45 human cases per year from 1999 to 2007 (www.ssi.dk). Approximately half of the human cases in Denmark were ascribed to nationally produced beef in 2006 (Anonymous, 2007). In other words, an estimated one to four humans die every year from eating *S.* Dublin contaminated beef produced in Denmark. It is also a complication sometimes seen in relation to other diseases, e.g. HIV and liver cirrhosis.

Surveillance and eradication programmes

In October 2002, a national surveillance program was initiated to monitor prevalence in the cattle population and to provide farmers with a tool to protect their herd when trading and interacting with other cattle herds. In January 2007, an eradication campaign was initiated by the Danish Cattle Federation with the aim of reaching prevalence close to 0% by the end of 2014. Thereafter, the number of new infections should below five outbreaks per year without further transmission of the infection. This is an ambitious goal supported by a wide range of activities both in the field and in research. In a report to the minister for Food, Agriculture and Fisheries submitted in October 2008 (unpublished to date) an estimate of more than 100 million DKK will be spent on surveillance of, intervention against and research about *S.* Dublin between 2009 and 2014.

1.2 Aim and outline of report

The aim of this report is to give an overview of scientific issues of importance for successful control and eradication of *S.* Dublin from the Danish cattle population.

This includes:

- pathogenesis of relevance for the diagnosis and epidemiology of the infection in cattle
- estimates of the validity of currently available diagnostic tests and examples of how to use and interpret the test results in relation to the purpose of testing
- epidemiology including prevalence, within-herd and between-herd transmission routes, risk factors and infection dynamics
- results from different intervention field trials that provide valuable knowledge for the forth-coming years of the eradication campaign
- perspectives, suggestions for further research and improvements to the national programmes

Other issues such as clinical signs and the pathology of the infection will only be briefly summarized in the pathogenesis section.

This report consists of seven sections:

1) first section provides the background for the report including the importance of *S.* Dublin for the cattle industry and food safety and definitions of terms used in the report
2) second section highlights important issues of the pathogenesis relevant for the epidemiology of *S.* Dublin in cattle and diagnostic test interpretation
3) third section gives an overview of available diagnostic tests and the validity and interpretation of test outcomes
4) fourth section concerns the epidemiology of the infection including within-herd dynamics and transmission between herds which is important to understand for both control and prevention of the infection
5) fifth section is about intervention against the infection in infected herds and reports results from field studies
6) sixth section gives an overview of the Danish *S.* Dublin programmes
7) seventh section are perspectives based on the previous sections

An interpretive summary is included at the end of each section.

Finally, this report offers suggestions for possible improvements to the current national surveillance program, intervention strategies and further research that directly or indirectly may be helpful to improve control and prevention of the infection. A list of references is provided at the end.

Definitions of terms used in this report

Throughout this report, the terms ”control”, “eradication”, “surveillance” and “intervention” are frequently used.

- **“Control**” implies activities aiming at reducing prevalence and preventing spread of the infection on herd, regional or national level.
- **“Eradication**” is defined here as reduction of herd prevalence to close to zero and discontinued spread of the infection between herds in Denmark.

These terms are often used in this manner in connection with control and eradication campaigns (Houe et al., 2006).

- **“Surveillance**” covers all activities that lead to recordings (monitoring) of infection *and* the consequences of such recordings, i.e. change in herd classification and trade restrictions.
- “**Intervention**” implies activities directed towards changing management of infected animals and environment to remove infection and prevent new infection from occurring. This may include use of decision tools to direct the intervention activities towards approaches with highest cost-benefit ratios.

Thurmond (2003) defined surveillance as “active, formal, and systematic processes intentionally directed to rapidly seek out and identify infectious disease agents or disease”. This definition fits a description of *active* surveillance components such as centrally organised screenings. *Passive* surveillance components such as sample submission upon clinical suspicion may also play an important part of surveillance for an infection – in particular low prevalence infection with clear clinical expressions. In Denmark, both active and passive surveillance components are combined in the current national surveillance program for *S.* Dublin in cattle.

2. Pathogenesis of *S.* Dublin

The pathogenesis of a disease is a combination of i) the mechanisms by which an etiological factor causes disease and ii) the development of the disease in the host. The word comes from the Greek *pathos*, "disease", and *genesis*, "creation". In this report the focus is on the development of disease and infection stages that are relevant for *S.* Dublin and control of this infection. Thus, it does not provide a thorough review of the abundant microbiological and molecular biological research that has been performed to improve understanding of cellular mechanisms of importance for the pathogenesis of *S.* Dublin and other *Salmonella* serotypes. It should be noted that despite enormous and world-wide research efforts to understand the mechanisms at cellular level we are still lacking a solid understanding of reasons and mechanisms for host adaptation, development of persistent infection and intracellular survival of *S.* Dublin (Uzzau et al., 2000; Olsen, 2005).

2.1 Uptake, dissemination and excretion of bacteria

After uptake, the outcome of *S.* Dublin infection in the host is highly dependent of several factors such as initial infection dose, immunity developed during previous infection, age and physiological state of the host. It is difficult to study the importance of all of these factors simultaneously so the knowledge we have today is brought together from both epidemiological field studies and experimental studies.

Uptake of bacteria

S. Dublin bacteria most commonly infect the host via oral uptake of contaminated food, water or milk or from contaminated environment, pen mates, calf or dam (Hardman et al., 1991). Less common infection routes include airways and conjunctiva (Nazer and Osborne, 1977; Wathes et al., 1988). These infection routes may be important if farm workers use high pressure cleaning when animals are still present in the barn. *S.* Dublin is also able to infect the foetus in utero. However, such infection will often lead to abortion or still born calves (Hinton, 1974; Hall and Jones, 1976). One study found prolonged carriers of *S.* Dublin after experimental inoculation through the teat canal (Spier et al., 1991). However, this is not believed to be a common infection route.

Infectious dose

The infectious dose required to cause clinical symptoms and pathological changes in the host is difficult to study in real life situations. State of the art therefore comes mainly from experimental infection studies. Peroral infection doses of more than 10^6 CFU usually lead to clinical signs and/or shedding in calves between 0 and 196 days of age, but the severity of the symptoms and pathological changes vary with age of the animal. The higher the infection dose the more consistently clinical symptoms can be reproduced (Taylor and Burrows, 1971a; Taylor, 1973; Nazer and Osborne, 1977; Robertsson, 1984; Steinbach et al., 1993; Silva et al., 2008). Calves in the milk feeding period (below 6 to 8 weeks) are highly susceptible (Nazer and Osborne, 1977; Segall and Lindberg, 1991). Studies in older cattle are few, but one experimental study in which intravenous inoculation of 0.1-1.4*10^{10} CFU was given to heifers aged 27 to 44 months reported severe illness (Hall and Jones, 1977). On the other hand, field studies of abortions report that abortion often occurs with few or no other clinical signs than transient pyrexia (Richardson and Watson, 1971; Hinton, 1974). This difference could be due to lower infection dosages under normal farm conditions and/or the fact that infection usually enters the host via the mouth and gastrointestinal canal and not intravenously. Hall and

Jones (1979) found that oral doses of 10^{10} or 10^{11} given to nine pregnant heifers gave variable responses from no clinical signs to severe illness with dysentery, pyrexia and abortions.

Invasion and dissemination

After colonisation of the gut, *Salmonella* bacteria adhere to and invade intestinal cells in the mucosa associated with the Peyer's patches through columnar enterocytes and specialized microfold enterocytes (M cells) and passes through to the lymphatic tissues beneath this layer. Here they enter macrophages that are drained to the local lymph nodes. This is an important barrier for further dissemination. If they move passed this barrier, the bacteria reach the lymph and blood (bacteraemia) and the reticuloendothelial tissue organs such as the spleen and liver while surviving and replicating inside the macrophages (Segall and Lindberg, 1991; Scherer and Miller, 2001). Some strains of *S.* Dublin have higher virulence meaning that they are better at passing the intestinal wall, the barriers in the lymphatic system and have a better intracellular survival than other strains (Selander et al., 1992; Watson et al., 1995; Wallis et al., 1995; Libby et al., 1997; Bäumler et al., 2000). This may explain some of the differences in clinical signs and course of outbreaks in cattle herds.

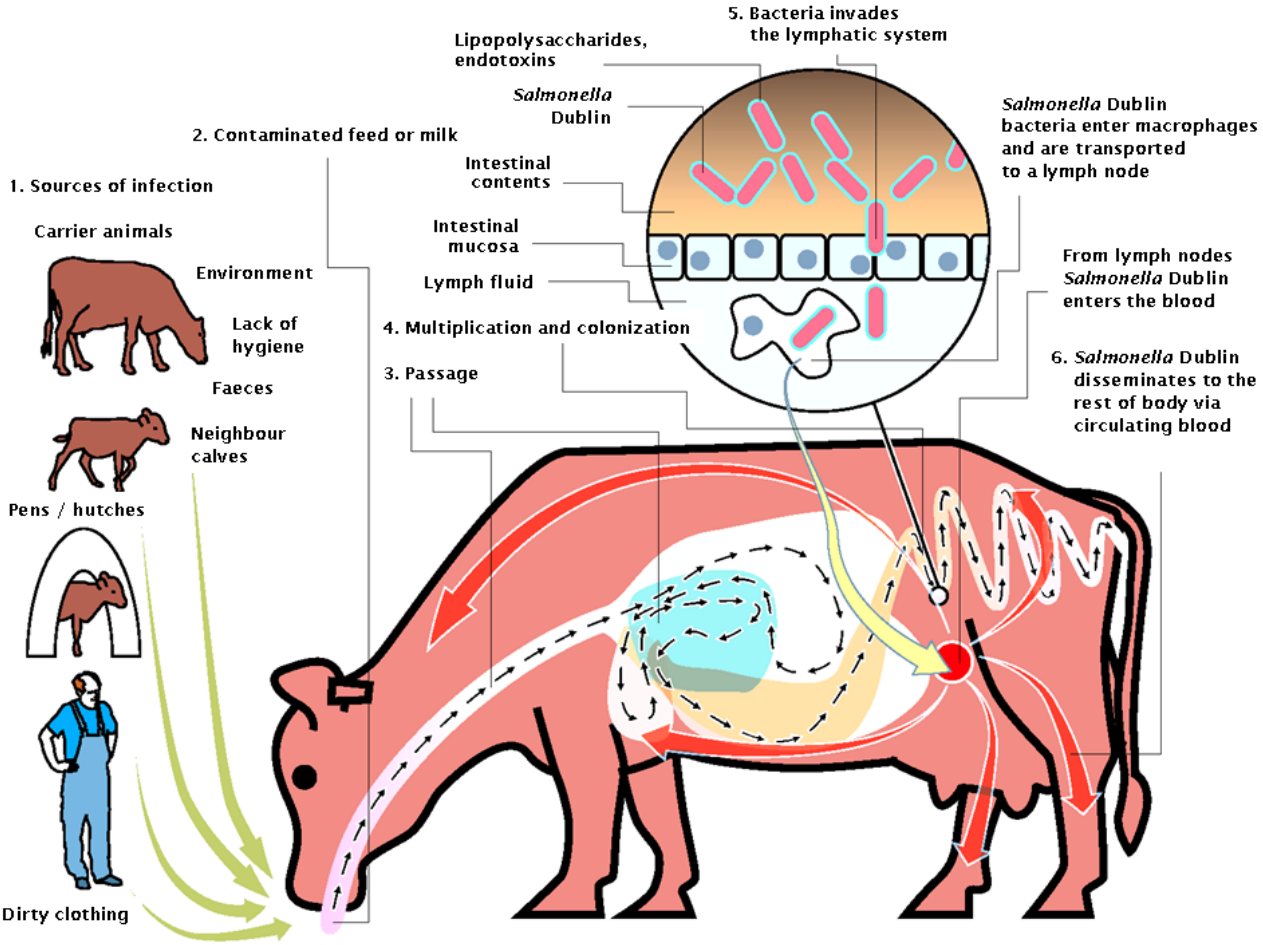

Figure 2.1 Pathogenesis of *S.* Dublin in cattle upon uptake of bacteria from contaminated milk, feed or environment. (Illustration: Lars Nørregaard, The Danish Cattle Federation)

Immune system responses to infection

At birth, the calf is immunosuppressed due to increased cortisol. Further, cell-mediated immunity is deficient at birth. Calves are therefore very susceptible to infections at this age. Around two weeks of age the cell-mediated immune capacity reaches levels similar to adult cattle. Therefore, the calves depend on antibodies, phagocytic cells and cytokines passively transferred from dam to calf through colostrum during the first days of life. The half life of passively transferred immunoglobulins in the neonate is between 11.5 and 16 days (Barrington and Parish, 2001).

The different components of the immune system, non-specific and specific (cellular and humoral immune systems), act in combination to combat *Salmonella*- infection. Inflammatory cells of the non-specific immune system such as macrophages, polymorphonuclear leukocytes (PMN), neutrophils, natural killer cells and their secreted cytokines constitute the first line of defence against invading *Salmonella*-bacteria (Vassiloyanakopoulos et al., 1998; Scherer and Miller, 2001).

Lymphocytes are the most predominant cell types of the specific immune system. After stimulation, B cells will develop immunoglobulins (antibodies) directed against *Salmonella* and present these on their cell surfaces. This may interact directly with the antigen and initiate a cascade of events in the primary immune response including production of clones of the original B cells into plasma cells and memory B cells. The first time cattle meet *Salmonella*-bacteria it may therefore take some time (5 to 7 days) before free specific antibodies will be circulating in the blood. In calves below the age of 11 weeks production of specific antibodies directed against LPS is poorer and slower (Da Roden et al., 1992). This is not only important in relation to immunity against the infection, but is also important because it explains why diagnostic tests based on measurements of antibodies directed against LPS will have poor sensitivity in young calves (Nielsen and Ersbøll, 2004).

Calves that have been infected previously will have some protection and thus show fewer clinical signs and have lower counts of bacteria in the small intestines upon experimental inoculation. However, the number of bacteria in the lymph nodes appears to be higher in calves that have previously been infected than in naïve animals (Steinbach et al., 1996). These findings may very well be related to an effective cell-mediated immunity, which is considered more important for protection of calves against *S.* Dublin infection compared to humoral immunity. In one study, it was found that calves with well-developed cell-mediated immunity had fewer and milder symptoms upon re-infection, and duration of bacterial excretion was shorter than in control calves. Calves with high levels of immunoglobulins originating from passive transfer did not have the same degree of protection (Chaturvedi and Sharma, 1981). While the cell-mediated immune response is important for the susceptibility to infection and ability to eliminate the bacteria from the organism, it is not necessarily correlated to the level of circulating antibodies (Robertsson et al., 1982). Also in older cattle, *S.* Dublin has been shown to survive in the mammary gland for more than a year despite high levels of specific anti-*S.* Dublin IgG (Spier et al., 1991). This indicates that the humoral immunity is not sufficient to clear this infection. In conclusion, antibodies may be a useful tool for diagnostics, but do not provide good information about the immune capacity of the animal for protection against the infection.

Experimental studies provide valuable knowledge about humoral immune responses upon initial infection. The IgM titre starts to increase approximately one week after inoculation and the IgG titre begins to increase about a week later in calves infected at the age of 6 to 7 weeks. The maximum titre of IgG is reached between 6 and 11 weeks after inoculation after which it gradually decreases and reaches baseline levels around 14 to 20 weeks after inoculation (i.e. between 2 and 3 months after peak IgG titre). It is possible that older cattle have faster IgG responses to infection (Robertsson, 1984; Smith et al., 1989; Da Roden et al.,

1992). One should keep in mind that in real life antibody concentration in blood and milk may not return baseline levels after transient infections, if the animal is exposed to *Salmonella*- bacteria repeatedly.

Excretion of bacteria

Bacteria may be shed trough most body excretions (urine, saliva, vaginal discharge and faeces), but faeces carries the highest numbers and are produced in larger amounts, thus being the most important vehicle of transmission from cattle (Richardson and Fawcett, 1973; Wray and Davies, 2000).

Faecal shedding of *S.* Dublin-bacteria begins between 24 hours and 7 days after uptake of the bacteria and lasts from one day to several months. The average is between 15 and 17 days in calves with clinical signs and probably shorter in older animals and animals asymptomatic infections (Taylor and Burrows, 1971a; Robertsson, 1984). In few cases, shedding may continue for years, usually intermittently. In such cases the animals are considered persistently infected, active carriers (Richardson, 1973b; House et al., 1993).

In an outbreak investigation in a Scottish dairy herd starting with an abortion in an adult cow, this cow was found to be shedding *S.* Dublin-bacteria in the vaginal discharges for three weeks. She was never found culture-positive in faecal samples (Mateus et al., 2008). Others have found *S.* Dublin-bacteria in organs of non-shedding cattle which lead to suggestions that *S.* Dublin can create latent carriers that may be important for persistence of infection in infected herds if they become reactivated (Richardson and Watson, 1971; Smith et al., 1989; Spier et al., 1991; House et al., 1993).

Sojka et al. (1974) provided one of the early studies of persistently infected cattle. One cow excreted between 2.4 x 10^4 and 4 x 10^5 CFU/g faeces and another cow excreted lower numbers (25 CFU/g to 1.4 x 10^4 CFU/g) for a period of at least 30 months.

In a Danish field study of cows in two endemically infected herds, semi-quantitative measurements of *S.* Dublin in faecal samples ranged from very low to low (0.2 to 1000 CFU/g) in test-positive cows. For comparison, a wide range of concentrations (0.2 - 10^8 CFU/g) was found in calves during an outbreak of *S.* Dublin in a dairy herd (Christensen, 2005).

Estimation of duration of infectiousness in calves in a field study

During 1999 to 2003, a large field study (known as the Kongeå-project) was carried out in the southern part of Jutland in Denmark. The project consisted of several PhD projects about infections of relevance for cattle. One of the nine PhD projects in the Kongeå-project dealt with *S.* Dublin epidemiology (Andersen et al., 2000; Nielsen, 2003). As part of the project we collected faecal and blood samples twice per week from calves and their dams in five endemically *S.* Dublin infected dairy herds. In one herd no animals were found faecal positive, so this herd was excluded from the calculations. Based on data from those sampling activities we were able to estimate the average duration of infectiousness (faecal shedding) and time to seroconversion upon infection in calves below the age of 180 days (Table 2.1). The estimate for mean duration of infectiousness was 17 days with wide variations from 3 to 68 days. The median was 10 days (Nielsen et al., 2007b). The results were used to model transmission parameters to gain more knowledge about *S.* Dublin infection dynamics within a herd as described in the epidemiology section of this report.

Table 2.1 Descriptive statistics for 19 calves (N) that were faecal culture positive for *S.* Dublin in four endemically infected Danish dairy herds. Nine animals that excreted bacteria did not show seroconversion in the study period (Nielsen et al., 2007).

Variables	N	Mean	Std.dev.	Median	Min-Max
Age at start of infectious period (in days)	19	40	23	43	3-70
Infectious period (in days)	19	17	19	10	3-68
Age at seroconversion (in days)	10	75	15	76	52-100
Time from start of shedding to seroconversion	10	36	17	28	11-67

Bacteriological culture of colostrum and milk samples

Farmers sometimes ask if colostrum and milk are important sources of infection and if it is preferable not to feed colostrum and milk from infected cows. There is one study that suggests that active carrier cows can excrete *S.* Dublin-bacteria in milk (House et al., 1993). However, there are not many studies supporting advice about milk-feeding during control of *S.* Dublin, but a few field studies have looked at it in Denmark. In the Kongeå-project, 128 cows were tested with faecal samples once or twice per week for 12 weeks after calving. In total, 15 cows shed *S.* Dublin in faeces, but only two were likely to be active carriers based on repeated shedding. We also collected colostrum samples from those cows immediately after calving. We attempted to obtain aseptically collected samples by washing the udder with iodine water and wiping the teats with alcohol wipes before stripping colostrum into a clean container while wearing clean gloves. We cultured 25 ml of milk per sample using conventional methods. None of these samples were found culture positive for *Salmonella*. In conclusion, colostrum is unlikely to be an important source of infection for calves (data unpublished). It was not possible to evaluate how many intermittent (latent) carriers were present in the herds as they are difficult to differentiate from transiently infected animals if they only shed once or a few times within a short interval. Such numbers have to be estimated from a mix of serological and bacteriological longitudinal studies as presented in the epidemiology section in this report (section 4).

In another Danish field study, five endemically *S.* Dublin-infected herds and one dairy herd with an acute outbreak of *S.*Typhimurium were visited several times during the autumn of 2006. Milk fed to calves was studied by collecting a sample of approximately 50 ml of milk directly from the milk bucket immediately after it was poured into the bucket at feeding time. It was recorded if the milk was milk replacer or milk from cows in the herd. The milk samples were tested by conventional culture methods. More than 100 milk samples were tested for *Salmonella* from these herds and none of them were found positive (Nielsen and Hansen, 2007).

Together, these two studies support the statement by Wray and Davies (2000) that European studies indicate that mammary-gland infection with *Salmonella* is uncommon. Thus, results from these two studies suggest that milk is not an important route of infection to consider during intervention against *S.* Dublin unless hygiene of stored milk to be fed to calves is obviously poor or direct faecal contamination of the milk is possible.

2.2 Infection stages

To gain an overview of the wide range of clinical expressions observed with *S.* Dublin-infection in cattle it is useful to refer to different stages of infection that an animal can experience upon infection (Richardson, 1973a; Robertsson, 1984; Wray and Snoyenbos, 1985; Rings, 1985):

Peracute infection – death will occur within 1 to 2 days after uptake of bacteria with few clinical symptoms after a short period of septicaemia and endotoxic shock. Such an infection is usually caused by very high doses in fully susceptible young calves, but may also be seen in adult cows or heifers in the beginning of an outbreak of *S.* Dublin-infection in a fully susceptible herd.

Acute infection – infection can be strictly enteric or systemic with transient bacteraemia. One or more of the following signs can be present: fever, depression, lack of appetite, pneumonia with respiratory distress, bloody or watery diarrhoea, arthritis and osteomyelitis leading to lameness and hot, swollen joints, meningoencephalitis leading to nervous symptoms in calves.
In adult cattle: Bloody or watery diarrhoea, fever, depression, abortion, sudden decreased milk production and lack of appetite.

Chronic infection – follows an acute infection and is mostly seen in animals older than 6 to 8 weeks that may show failure to thrive, bloody and loose stool, intestinal casts, slightly elevated temperature, and scruffy hair coat and growth retardation. Often lameness is part of the clinical picture due to arthritis or osteomyelitis. Necrosis of the skin on ears, tail or distal limbs may also be seen. The clinical signs are caused by tissue damage from previous or current infection, and the animal may or may not be shedding bacteria.

Asymptomatic, persistent infection – can occur in all ages of cattle and follows acute infection with or without clinical signs. The animal carries *S.* Dublin-bacteria in internal organs, lymph nodes and sometimes also the intestinal wall. Shedding of bacteria may occur through milk or faeces, though bacteria shed in milk is not thought to be commonly seen in Europe (Wray and Davies, 2000). Some state that stressful events may cause reactivation of a latent infection (Spier et al., 1991). However in a recent experimental study in Denmark we were unable to reactivate latent infections in heifers and cows by experimental immunosuppression (Lomborg et al., 2007). It may be that persistently infected carriers could be either active with continuous or intermittent shedding or latent with rare reactivation of infection and that it is mainly the active carriers that are important to the infection dynamics of *S.* Dublin in infected herds.

It should be noted that the terminology of different types of carriers is not universal (Smith et al., 1989; Smith et al., 1992; Wray and Davies, 2000; Veling, 2004; Nielsen et al., 2004a). Most agree that “active carriers” are frequently or continuously shedding bacteria, and that “passive carriers” can transfer the bacteria through the gut without becoming clinically or pathologically affected. However, the term “latent carrier” covers anything from a non-shedding infected animal that may or may not become reactivated and start shedding to an animal that sheds bacteria intermittently and therefore should be considered fairly high-risk animal regarding transmission of infection within and between herds.

2.3 Host and agent factors of importance for the pathogenesis

Host adaptation

S. Dublin is host-adapted to cattle. This means that it is mainly found in cattle and most frequently causes disease in this species (Wray and Sojka, 1977; Selander et al., 1992). This does not mean that other animal species and humans cannot become infected and ill from the infection but it is not commonly seen. Exactly why *S.* Dublin is host-adapted is still a subject of debate. Several authors have come up with different hypotheses but so far there is no general agreement about the underlying mechanisms. Hypotheses include differences in evolution of so-called *Salmonella* pathogenicity islands (Bispham et al., 2001; Eswarappa et al., 2008). Some other *Salmonella*-serotypes are also host-adapted (or host-restricted) to other animal species or humans. In a review it was underlined that the host-specificity is most likely caused by a unique sets of mechanisms for each different serotype rather than a common explanation for the host specificity phenomenon as such (Uzzau et al., 2000). In practice, the mechanism for host adaptation may not be important for control of the infection, unless it is linked to virulence or the ability of the bacteria to cause persistently infected intermittent shedders and can somehow be controlled. However, the fact that *S.* Dublin is host-adapted makes it feasible for the cattle industry to control it “within own walls” so to speak. Even though *S.* Dublin is host-adapted it can still lead to infections in other species including humans, but it is most commonly seen in cattle.

Virulence factor and bacterial genes

Most experimental studies of *Salmonella*-pathogenesis have been performed in chickens, rats or mice which are not the ideal species to use when examining mechanisms of *S.* Dublin-pathogenesis in cattle. Very often general conclusions about *Salmonella*-pathogenesis are based on studies of other serotypes than *S.* Dublin. *Salmonella* virulence plasmid genes (spv genes) of the bacteria are considered important for the pathogenesis of *S.* Dublin (Libby et al., 1997). However, one study found that spv genes may only be needed for *S.* Dublin to produce systemic disease, but are not necessary for enteric disease to occur in cattle (Wallis et al., 1995). The ability to multiply and survive intracellularly is probably important for the tendency of *S.* Dublin to produce prolonged carrier states (Brackelsberg et al., 1997; Chadfield et al., 2003). Whereas the area is important for progress in our understanding of the complex *Salmonella*-pathogenesis, state of the art of molecular biology for *S.* Dublin today leaves us with conclusions that are not directly transferable to control or prevention strategies yet. In practice the research has given us some explanations for the variation in clinical and pathological expressions of the disease and for variations in diagnostic test validity for different strains of *S.* Dublin in different hosts and different herds.

Lipopolysaccharides and endotoxic shock

Outer membrane lipopolysaccharides (LPS) that present O-antigens to the environment around the bacteria are important for the pathogenesis of *S.* Dublin (Rycroft, 2000). They are also relevant in diagnostic tests. Immunoglobulins (Ig) from the host are directed against the O-antigens and the response is serotype-specific, because different serotypes present different O-antigens on the bacterial surface. However, some *Salmonella*-serotypes have common O-antigens, so some cross-immunity may occur upon infection with such serotype (Konrad et al., 1994). For instance, cattle infected with *S.* Dublin usually produce immunoglobulins directed against LPS O-antigens 1, 9 and 12. *S.* Typhimurium may present O-antigens 1, 4, 5 and 12, so the two serotypes have O1 and O12 in common which may lead to both cross-immunity and cross-reactions in serological tests (Konrad et al., 1994; Mohler et al., 2006).

LPS are also important because they contain lipid A, a structure that is a so-called “endotoxin”. Even extracted from the bacteria this component is toxic to host cells through induction and release of cytokines from monocytes and macrophages. Interferon, tumour necrosis factor, colony-stimulating factor and interleukin 1 are all examples of cytokines released during *Salmonella*-infections. They contribute to vascular damage and thrombosis and thus play an important role in the tissue damage leading to fever, disseminated intravascular coagulation, circulatory collapse characteristic for “endotoxic shock” during *Salmonella*-infections (Rycroft, 2000).

Genetic host factors

Genetic host factors also play a role in how *S.* Dublin infection develops upon uptake. Expression of the host gene “Nramp1” has been mentioned as an important determinant of pro-inflammatory cytokine expression which affects the level of invasion and survival in macrophages and clinical symptoms in relation to *Salmonella*-infection (Valdez et al., 2008). Also an *Lps* locus in the host seems to regulate the host’s ability to respond to LPS on the bacterial surface (Scherer and Miller, 2001). Differences in expression of such genes may partly explain some of the susceptibility differences between individual animals. Finally, the physiological state of the host is important for the development of infection as described in more detail below.

Physiological host factors

In weaned calves and adult animals, the physiological states of the rumen and the rest of the gastrointestinal tract are very important for multiplication and colonisation of the gut which precedes invasion of the intestinal epithelium. Volatile fatty acids and a normal pH below 7 in the rumen and the low pH (<4.8) in the abomasum usually inhibit multiplication of *Salmonella*-bacteria (Chambers and Lysons, 1979; Mattila et al., 1988). Furthermore, normal peristalsis and competing microflora of the rumen and small intestines prevent adhesion to the epithelial cells. Thus, it takes either a sufficiently high infection dose or a disruption of the normal function of the gastrointestinal tract to allow *Salmonella*-bacteria to multiply, colonize and invade the epithelium in the small intestine. Such disruption may occur during starvation, deprivation of water, transportation, other diseases, sudden changes in feeding strategies, feeding of poor quality feed, severe weather conditions and antibiotic treatment (Chambers and Lysons, 1979; Mattila et al., 1988; Morisse and Cotte, 1994; Wray and Davies, 2000). Also, concomitant infections such as Bovine Virus Diarrhoea (BVD) and *Fasciola hepatica* can aggravate *S.* Dublin infections or make the host more susceptible to becoming infected (Aitken et al., 1978; Aitken et al., 1981; Wray and Roeder, 1987).

2.4 Repeated antibody measurements in individual animals

In one part of the Kongeå-project, samples of blood and faecal matter were collected twice a week from calves for approximately 12 weeks in five endemically infected dairy herds. This included a precolostral serum sample. It was recorded if the animals received *S.* Dublin serum treatment (Nielsen, 2003). The graphs in Figure 2.2a and 2.2b illustrate examples of ELISA measurements of antibodies directed against *S.* Dublin LPS and faecal culture positivity in calves that did and did not receive *S.* Dublin serum treatment, respectively. Apparently, the measureable ELISA response is very similar in calves that have received colostrum from a dam with specific antibodies and calves that have received *S.* Dublin serum treatment once shortly after birth. The ELISA response is given as ODC% which is the background corrected optic density in relation to a known positive control sample.

Chaturvedi and Sharma (1981) and Robertsson et al. (1982) found that the level of circulating antibodies do not indicate much about the level of protection of the calf. Hence, it cannot be recommended to rely solely on prevention of new infections by passive transfer of antibodies during intervention efforts.

In Figure 2.2c the humoral immune response is shown for a calf that was infected shortly after birth and did not receive any *Salmonella*-specific antibodies via colostrum. This calf was clinically ill with salmonellosis. Figure 2.2d is an illustration of how the humoral immune response can vary over time in a cow that presumably became infected several times during the study period.

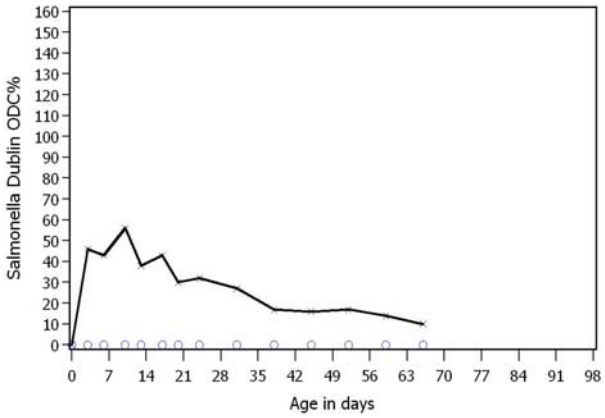

Fig. 2.2a Antibodies (solid line) in a calf that has received *S.* Dublin serum treatment immediately after birth. Blue circles indicate that faecal samples were tested for bacteria. No *Salmonella*-bacteria were found in these samples. The first measurement is precolostral.

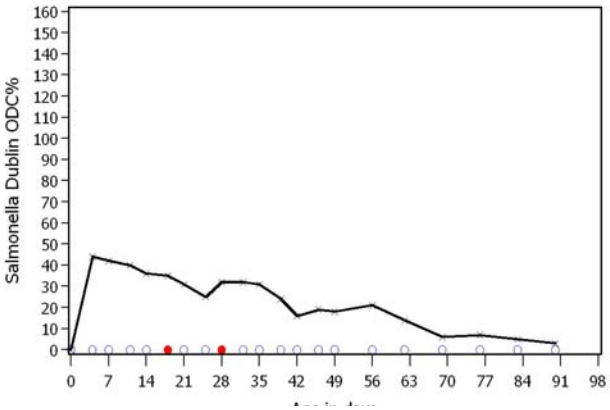

Fig. 2.2b Antibodies (solid line) and faecal excretion (red dots) in a calf that has received *S.* Dublin-specific antibodies through colostrum from the dam within 6 hours after birth. Blue circles indicate that faecal samples were tested for bacteria. The first measurement of antibodies is precolostral.

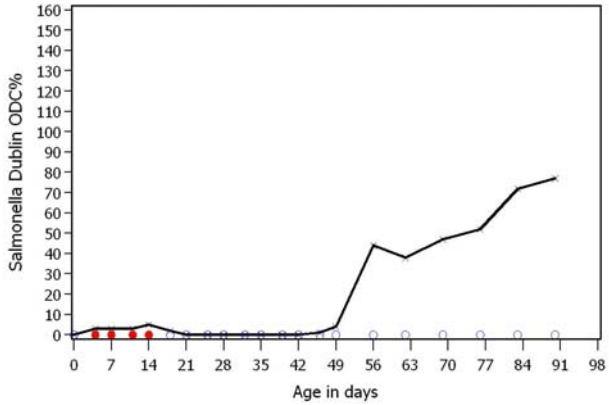

Fig. 2.2c Antibodies (solid line) and faecal excretion (red dots) in a calf that was infected shortly after birth. Blue circles indicate that faecal samples were tested for bacteria. The first measurement of antibodies is precolostral. This calf did not receive specific antibodies from the dam.

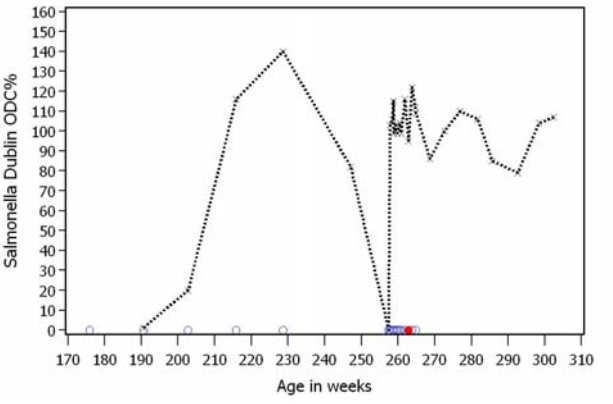

Fig. 2.2d Primary and secondary (or later) humoral immune responses in SDM-cow. The red dot indicates faecal excretion. Blue circles indicate that faecal samples were tested for bacteria and found negative.

2.5 Interpretive summary of the pathogenesis section

Most knowledge about *S.* Dublin-pathogenesis comes from experimental studies. Unfortunately species used in the experiments have not always been relevant for ruminants. Hence, a lot of work has been done, but we are left with only a few major and consistent conclusions relevant to control and eradication of the infection in practice from such studies: i) cell-mediated immunity is apparently more important for protection of cattle against *S.* Dublin infection compared to humoral immunity and ii) antibodies may be useful for diagnostics, but do not provide sufficient immune capacity of the animal to protect against this highly invasive infection. However, methods to improve cell-mediated immunity, such as vaccinations, are unlikely to be profitable in the long run, because they only provide partial protection. Clinical signs and mortality are reduced, but shedding of bacteria still occurs and vaccination often has to be done repeatedly, so the expenses become high.

Valuable information about dynamics of antibodies in individual cattle upon infection is available from experimental studies. The IgG titre begins to increase about two weeks after uptake of bacteria in calves. The maximum titre of IgG is reached between 6 and 11 weeks after uptake and thereafter gradually decreases and reaches baseline levels around 14 to 20 weeks after inoculation (i.e. between 2 and 3 months after peak IgG titre). It is quite possible that older cattle have faster IgG responses to infection. However, one should keep in mind that in real life such a clear cut picture is rarely seen, because antibody concentration in blood and milk do not return to baseline levels after transient infections, if the animal is exposed to *Salmonella*-bacteria repeatedly.

Duration and concentrations of excretion of bacteria is highly variable upon uptake. The average duration of infectiousness is around 2 weeks in calves, probably shorter in adult and re-infected cattle. The concentration of excreted bacteria in faeces varies from very low numbers (< 0.2 CFU/g) to very high numbers (>10^8 CFU/g) depending on age and within-herd prevalence and transmission patterns in the herd. Colostrum and milk are not likely to be common direct sources of infection, but milk may become contaminated with infected faecal matter during milking or storage before being fed to calves.

The wide variations in the infection course and clinical expression of *S.* Dublin-infections in cattle make it challenging to communicate advice to farmers about how to control the infection. Often advisers seek clear-cut recommendations that can be easily communicated. Farmers seek simply advices similar to those used in previous eradication campaigns such as the BVD eradication campaign. Test-and-cull-procedures were central in the BVD eradication campaign. However, for *S.* Dublin the fairly simple recommendation from the experimental studies about culling of carrier animals detected by repeated antibody measurements need to be used with care. Due to the faecal excretion and survival outside the host, *S.* Dublin has an environmental component that needs to be taken into account and animal classified as carriers based on repeated antibody measurements are not all shedders. Thus, culling strategies might have poor cost-benefit ratios.

Even though the pathogenesis of *S.* Dublin is still intriguing and academically interesting, it is unlikely that the lack of thorough understanding of the pathogenesis of *S.* Dublin at molecular level is an important hindrance of successful intervention in cattle herds today.

3. Diagnostic tests

Critical to most infections in humans and animals is “diagnosis”. The definition of diagnosis varies depending on the source of information, but most agree that it comes from Greek. The word means to distinguish or decide through (*dia*-) knowledge (*-gnosis*). Some dictionaries emphasise that diagnosis has two components; i) a critical analysis of the nature of the something and ii) the conclusion reached by such analysis. Thus, interpretation may be involved in making a diagnosis. A diagnosis can only be made with a stated purpose in mind. If the purpose is to figure out what a certain set of symptoms in an animal is caused by, the etiological agent may be important to isolate and relate to the symptoms. Often, other possible etiological agents have to be ruled out as a causing factor in such cases. This is part of the analysis. If the results of the analysis are not able to rule out all other possibilities than one and at the same time confirm that one possibility with great certainty, a decision has to be made to decide how to interpret the result. The purpose of making a diagnosis could also be to determine the state of infection (not whether or not the animal is infected, but whether it is acutely infected, chronically infected, latently infected or previously infected but now recovered). Such a diagnosis can be important for e.g. prognosis and treatment.

Clinical and pathological signs are often too unspecific to diagnose. To aid in making a diagnosis technical tools “diagnostic tests” can be useful. Using one or more diagnostic tests is part of the analysis to gain more knowledge and get a better ground on which to make a distinction. Diagnostic tests can be anything that increases our knowledge about the condition in question, e.g. clinical examinations, measurements of antibodies, culturing of bacteria, scoring of body condition, observations of feed intake etc. The process of making a diagnosis can therefore become quite complex. In this section of the report, only diagnostic tests related to detection of *S.* Dublin-bacteria and measurements of antibodies by ELISA in serum and milk samples will be covered. This does not mean that other diagnostic tests cannot be relevant for *S.* Dublin, but currently there are no other types of tests for *S.* Dublin on the market. Clinical examinations may be helpful in increasing the sensitivity of bacteriological detection by selecting for animals most likely to carry and shed bacteria.

3.1 Detection of the bacteria

Detection of bacteria in faecal, organ or environmental samples can be done via conventional bacteriological culture which has the advantage of being able to identify the type of *Salmonella* in question. The disadvantage is a fairly low sensitivity. This may be due to poor growth potential or that the bacteria are present in very low numbers in the sample. Newer techniques are based on detection of genetic material from the bacteria (PCR-techniques). These are most likely more sensitive, but have the disadvantage that we are not always able to grow the bacteria for typing even if the sample turns out positive in the PCR-test. If the detected bacteria cannot be grown in culture we will not be able to determine the serotype. It is sometimes difficult to know if the bacteria detected in the sample were able to transmit to other animals or not. This section reviews some studies that have tried to evaluate the methods currently in use or being developed for detection of *S.* Dublin-bacteria in Denmark.

Conventional bacteriological culture

Conventional bacteriological methods are based on a number of steps aiming at isolating bacteria in the sample. These include pre-enrichment, selective enrichment, plating and confirmation (Baggesen et al., 2007). The methods require that bacteria in the sample are able to grow in the enrichment steps. If this is the case, the method should in principle be able to detect as little as one CFU in the sample. However, in

practice samples with fewer than 10 CFU/g are not consistently found positive with this method (Baggesen et al., 2007).

Richardson and Fawcett (1973) stated that rectal swabs are less sensitive than culture of faecal samples. This is due to the small amount of material used for the test. However, if the concentration of bacteria is higher than 100 CFU/g even rectal swabs should be fairly sensitive (60-100%). The authors recommended that the number of tested animals should be high to obtain a positive herd diagnosis based on bacteriological cultures of faecal material in infected herds. Their conclusions are in line with other studies that show that faecal culture has poor sensitivity for detection of *S.* Dublin-infected animals or herds. Individual animal sensitivity was between 6% and 14% in cattle without clinical signs when using pooled faecal samples with individual follow-up on positive pools (Nielsen et al., 2004b). Herd sensitivity was 38%, if herd diagnosis was based on testing animals with recent clinical signs of salmonellosis (Veling et al., 2002).

Possible causes for poor sensitivity may be intermittent shedding in infected cattle (House et al., 1993; Nielsen et al., 2007b) or low concentrations shed by cattle without clinical symptoms or repeated infections (Sojka et al., 1974; Steinbach et al., 1996). In a master thesis project conducted in Denmark during 2004 and 2005, shedding of *S.* Dublin in cows in two endemically infected herds was studied. In total, 58 cows were sampled twice daily for four consecutive days, four times with three-week intervals. All positive cultures were analysed in ten-fold dilutions to obtain semi-quantitative estimates of the concentration of bacteria in the sample. Thirteen cows were found to be shedding bacteria at least once. Concentrations were below 10 CFU/g in 15 out of 18 positive samples. Two contained 10-100 CFU/g and one contained between 100 and 1000 CFU/g. All cows were sampled approximately 30 times and only two cows shed bacteria more than once (two and four times, respectively) (Christensen, 2005). In the same project, calves in three *S.* Dublin outbreak herds were sampled up to four months after the outbreak. In one herd shedding had ceased when the project sampling began. In another there were only very few shedders. In the last herd, *S.* Dublin was present in high numbers and with high prevalence of positive faecal cultures. In this herd, the concentration of bacteria in the faecal samples ranged from <0.2 to $>10^8$ CFU/g. Two calves out of 27 were culture positive at every sampling round over a period of more than four months (Christensen, 2005). Together the studies suggest that one should expect the performance of the faecal culture test to be quite different depending on the scenario for which it is used. Further, herd prevalence and transmission patterns can play a major role for the sensitivity of the test at herd level.

Studies of *Salmonella* in pig faeces have shown that the bacteria may have a tendency to cluster in the sample (Cannon and Nicholls, 2002) a phenomenon which may be even more pronounced in cattle faeces. Also, the weight of the sample may play a role though it is not clear if it is always so that a larger amount will increase the sensitivity (Funk et al., 2000; Cannon and Nicholls, 2002; Champagne et al., 2005; Baggesen et al., 2007). In an experimental study on spiked faecal samples we found that the origin of the faecal material and the *S.* Dublin-strain had significant influences on the performance of the culture test. This suggests that faeces may contain growth inhibiting factors to varying degrees (Baggesen et al., 2007). This could for instance be influenced by feed type and competing microflora in the herd.

Predictive values are usually influenced by the underlying prevalence of infection. The positive predictive value for faecal culture of *S.* Dublin can be assumed to be 100%. This means that if the test is positive we can be 100% certain that the animal was shedding bacteria in faeces. This is independent of the underlying prevalence. The estimated negative predictive values of individual faecal culture at different prevalences of infection in the herd were estimated by Nielsen et al. (2004b) based on data from the Kongeå-project. The estimates varied from 0% to 13% and were highest at the lowest underlying prevalence. This means that if a sample is negative, it does not tell us much about the true infection status of the tested animal. The culture

method used for that study was pools of five individual samples. If the pool was positive the individual samples were cultured to detect the positive animal(s) in the pool. Direct culture of individual faecal samples might give slightly better sensitivity and negative predictive values, but it doesn't change the fact that a negative test results leaves us with little new knowledge about the infection status of the tested animal.

Faecal pools

Often it is desirable to collect samples from several animals or pens to attempt save resources while trying to obtain similar or better herd sensitivity. Evaluation of the effect of pooling faecal samples is not trivial, because many factors such as weight and number of samples included in a pool, the variation in bacterial concentrations in the samples and the underlying prevalence in the herd may affect the herd sensitivity upon pooling. A model has been developed to estimate the herd sensitivity given one knows the distribution of bacteria or can make reasonable assumptions based on previous studies. For *Salmonella* spp. in cattle faeces, the model showed that a reduction in the assumed prevalence of shedding can cause a substantial reduction in herd sensitivity when using pools compared to individual samples. However, the herd sensitivity was much less sensitive to changes in prevalence when the number of samples per pool was high, or when the number of pools per herd-test was high, or both (Jordan, 2005). It would be relevant to try to run this model for the specific case of *S.* Dublin in cattle using field study distributions of within-herd prevalence and bacterial concentrations such as those found in field studies in Denmark where the situation is obviously very different in the outbreak situation than in endemically infected herds (Christensen, 2005).

In the Kongeå-project, faecal samples were analysed both in pools of five (5 x 5 g) and individually (1 x 5 g) where possible during autumn 2001. The results were that 43% (19 out of 44) of pools containing faeces from at least one individual faecal culture positive animal were positive (Nielsen, 2003). On the other hand 490 pools were negative out of 492 pools that were composed of faeces from animals that were negative in the individual samples. Assuming that the specificity of faecal culture is 100% this shows that the sensitivity of the individual faecal sample method is not perfect either. This may explain to some extend why the sensitivity of faecal culture was estimated to be so low in the study by Nielsen et al. (2004b). The test results used for that study were based on a "pool-first" method in which individual samples were pooled into pools of five, and only if the pool was positive would the individual samples in the pool be tested to obtain a diagnosis on animal level. It does, however, not change the fact that faecal culture sensitivity is probably below 25-30% in non-clinical cases.

Polymerase chain reaction (PCR) tests

Conventional culture methods are expensive and time consuming, and today faster and more sensitive methods are being developed for detection of *Salmonella*-bacteria in food products and environmental samples, but also faecal samples (Kongmuang et al., 1994; Fratamico, 2003). The studies suggest that techniques based on detection of genetic material (DNA) are more sensitive than the traditional culture methods. However, published studies only tested samples that were under suspicion for being *Salmonella*-infected, but lacked proper negative reference groups. It is therefore difficult to evaluate how much the sensitivity is improved by these methods and if specificity is an issue that should be considered (Berreda, 2006).

There are two main principles in polymerase chain reaction (PCR)-methods: the traditional PCR and real-time PCR. In traditional PCR, *Salmonella*-specific genes (target DNA) in the sample becomes bound to a primer that is complementary to the target DNA of interest. A thermo cycler amplifies the DNA through three steps (denaturation, annealing and extension) usually over 30 or more cycles in which gene sequences are

copied. In each cycle, the numbers of DNA copies are doubled. Then the product is analysed on an agarose-gel to see if the cycles have resulted in copies of the relevant gene and of the right size. With this method there is a risk of contamination of the samples when the reaction tubes are opened to run the agarose-gel test. The test result is qualitative (yes/no) and it does not provide information about which serotype of *Salmonella* was detected in a positive sample.

In real-time PCR (or quantitative PCR) the amount of copied DNA is counted by a computer after each cycle by the use of fluorescent probes. It is not necessary to test on an agarose-gel. A so-called dual labelled probe in annealed a specific location between two specific primers adding more specificity to the test. The probe emits fluorescence through a reporter dye when it is cleaved by polymerase. This happens when the primer becomes extended in the presence of target DNA. With each cycle additional reporter dye molecules are cleaved leading to increased fluorescence. The higher the initial concentration of target DNA in the sample the sooner a significant increase in fluorescence can be measured by the computer and displayed by the software. The first cycle where fluorescence becomes higher than the background is called the threshold cycle (Ct). The Ct-value is therefore inversely correlated to the starting concentration of target DNA in the sample. The performance of PCR-tests depends a lot on well functioning primers and probes, and internal controls are important.

In a master project samples from suspected *S.* Dublin infected animals and herds were tested by the use of both conventional bacteriological culture and real-time PCR (TaqMan®) using a 94 bp amplicon on the ttr locus in the *Salmonella* genome (Berreda, 2006). In one set of samples originating from cows and heifers with persistently high serology (suspected carrier animals) the PCR-test found eight faecal samples from two animals and one milk sample from a third animal positive for *Salmonella* where the conventional culture method did not find any of those positives. Also, the PCR found nine organ samples (liver, mammary lymph nodded, caecal lymph nodes, caeum, colic lymph nodes, and tonsillar tissue of the colon) from four animals positive where the conventional culture only found four organ samples positive from three of the same animals.

In another set of samples from endemically infected cattle herds, 18 slurry tanks in nine herds were sampled three times with approximately five weeks intervals. At each sampling three samples were collected from each tank. The PCR found 87 whereas the conventional culture test found 52 positive tank samples. Six of the culture positive samples were PCR-negative and 41 of the PCR-positives were culture negative (Berreda, 2006). No known negative control samples were included in the study, so it is difficult to know if the specificity of the PCR was good.

In the same master project, development of a serogroup D-specific *Salmonella* real-time PCR was attempted. If such a test could be developed it would be of great interest for the surveillance and eradication programmes for *S.* Dublin in Denmark, in particular in the later stages of the eradication programme. However, difficulties were encountered designing primers and probes that could be used in the serogroup D-specific PCR and at termination of the project the sensitivity of the developed serogroup D-specific PCR test was not satisfying and positive test results were difficult to reproduce. More work is warranted if such a test is to become relevant for routine screening or confirmation of clinical outbreak in the future (Berreda, 2006).

3.2 Measurements of antibodies

In the pathogenesis section of this report is was described how the specific immune response becomes activated when animals are infected with *S.* Dublin-bacteria. The humoral immune response is used as an

indicator of current or previous infection by the use of enzyme linked immunosorbent assays (ELISAs) measuring the immunoglobulin-G levels in blood and milk directed against O-antigens from *S.* Dublin. The methods may vary slightly between laboratories. ELISAs have poor sensitivity early in the infection period, primarily the first time an animal becomes infected, because it takes some time for the immunoglobulin levels to rise to measurable levels, but they are useful tools for surveillance, management support and evaluation of intervention efforts as will become evident in this section.

Bulk tank milk ELISA

In most Danish dairy herds milk is collected every day or every second day. Bulk tank milk samples are routinely collected by dairy truck drivers from all dairy farms either every time milk is collected or weekly depending on the dairy company. The milk is tested for somatic cell counts, fat, protein and urea contents. Every three months a bulk tank milk sample from this quality control scheme is tested for antibodies in the national surveillance programmes for *S.* Dublin, Bovine Virus Diarrhoea (BVD) and Infectious Bovine Rhinotracheitis (IBR). In a high risk region for IBR (southern part of Jutland) bulk tank milk is tested every month. Bulk tank milk is a convenient pooled sample from dairy herds and it facilitates very cheap surveillance options. However, it has some limitations because the milk from infected (or high-titre) animals is diluted by the milk from non-infected (or low-titre) animals. The dilution effect may be different in small herds compared to large herds. Also, bulk tank milk only includes measurements on cows, so it does not necessarily say much about infection in young stock. This is particularly important in herds where young stock and cows are kept separated in different buildings.

In one study, we tried to estimate sources of variation in *S.* Dublin bulk tank milk antibody measurements (Nielsen and Ersbøll, 2005). Repeated bulk tank milk antibody measurements from 31 herds were analysed for associations with herd factors in three models. The model that best described the variation in bulk tank milk antibody measurements included *Salmonella* bacteriological status (positive or negative for *S.* Dublin or *S.* Typhimurium) after faecal sampling of all animals and barn environment), mean yield-corrected antibody measurements in individual cows and number of high titre cows (>80 ODC%). This model explained 45% of the variation in bulk tank milk antibody measurements. The study tells us that the level of antibodies in bulk tank milk is strongly associated with the activity of infection among cows (current or recent infection). The model also suggested that other (unknown) herd factors are important. In Fig. 3.1 it is illustrated that prevalence of cows with high (>80 ODC%) ELISA values in individual milk is important for the bulk tank milk *S.* Dublin ELISA response.

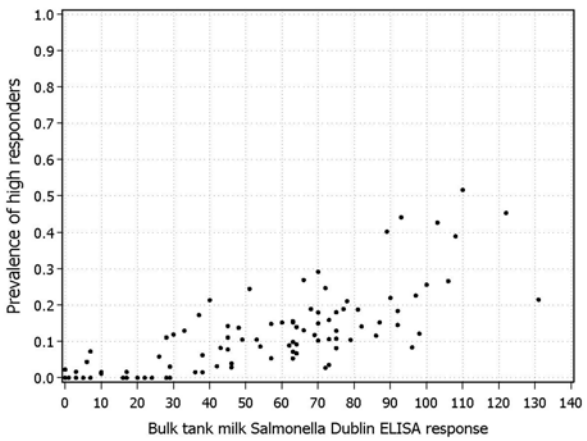

Figure 3.1 Prevalence of high responding cows (ODC%>80) vs. bulk tank milk *S.* Dublin ELISA response

Veling et al. (2001) found that the sensitivity of a bulk tank milk LPS ELISA similar to the one used in Denmark was 38% at cut-off point OD=0.4 which is approximately equivalent to 25 ODC% often used in Denmark. In total, 30 of 79 dairy herds known to be infected with *S.* Dublin were positive in bulk tank milk collected 2 to 4 months after the initial outbreak of *S.* Dublin was recognized. At lower cut-off the sensitivity was higher, but even at the lowest cut-off OD=0.1 the sensitivity of the bulk tank milk ELISA was only 68.4%. The specificity was estimated in 125 Dutch control herds that had no history of salmonellosis and 200 Swedish herds from an area of Northern Sweden that had no history of *S.* Dublin outbreaks. The specificity was 98.4% in Dutch control herds and 100% in Swedish control herds at cut-off OD=0.4. Further work in these herds found that herd-level sensitivities (HSe) of different testing procedures as shown in Table 3.1. It appears that bulk tank milk has a higher sensitivity compared to culture methods and can be improved by combining with serology from a good indicator group of young stock for herd classification. However, specificities of these combination methods have not been evaluated. In those studies, the HSe was evaluated as a one-time event. Improved herd level sensitivity can be obtained by combining repeated measurements of bulk tank milk over time. This is what is done in the Danish national surveillance program and the method was evaluated by Warnick et al. (2006). It was estimated that the HSe was 95% and HSp 96% at 15% national prevalence. We are currently trying to develop an early-warning system based on register-data from the Danish Cattle Database to improve early detection of *S.* Dublin outbreaks in dairy herds.

Table 3.1 Herd sensitivity (HSe) for different herd testing procedures (Veling et al., 2002; Warnick et al., 2006)

Herd testing procedure	HSe
Bulk tank milk LPS ELISA at cut-off OD=0.4	38%
Culture of dung-pits	45%
Drinking water cultures	5%
Bulk tank milk filter cultures	7%
Faecal culture of animals with current or earlier signs of salmonellosis	38%
Serology of all young stock	100%
Serology of all young stock between 4 to 6 months	91%
Serology of animals with current or previous signs of salmonellosis	80%
Combination of bulk tank milk ELISA and serology of animals with current or previous signs of salmonellosis	91%
Combination of bulk tank milk ELISA and serology of all young stock between 4 to 6 months	99%
Combination of bulk tank milk ELISA in four samples collected over 5 to 12 months	95%

Since the true herd status is unknown in herds most of the time, the predictive values of such herd testing programmes are more interesting. The positive predictive value (PPV) denotes the probability that a herd is truly infected given that the herd test procedure gives a positive result. In the surveillance program for *S.* Dublin this corresponds to Level 2 based on too high antibody levels in bulk tank milk. PPV was estimated to 80% at 15% national prevalence, meaning that 20% of herds in Level 2 are probably not infected at the time the herd classification is determined. The proportion of false positive herds will increase as prevalence is reduced in the country. The negative predictive value (NPV) gives the probability that a herd is truly non-infected given that the test procedure gives a negative result. This corresponds to Level 1 in the national surveillance program for *S.* Dublin. NPV was estimated to be 99% (Warnick et al., 2006), meaning that 1% of Level 1 herds are truly infected even though they do not (yet) test positive. The proportion of false negative herds will decrease as prevalence reduces nation-wide. Figure 3.1 shows an example of bulk tank milk ELISA measurements from a herd that experienced a *S.* Dublin outbreak in winter 2001 and later participated in an intervention study to eradicate the infection from the herd.

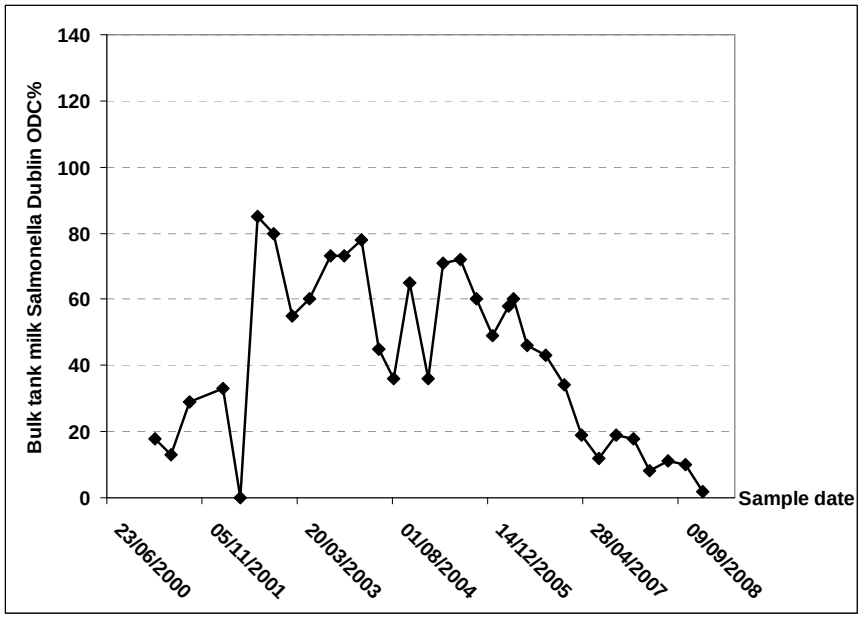

Fig 3.1 Bulk tank milk antibody ELISA measurements in a dairy herd that had an outbreak of *S.* Dublin in winter 2001.

Individual milk ELISA

Today, approximately 90% of all dairy herds in Denmark participate in a voluntary milk production control scheme “ydelseskontrollen”. They have samples collected from individual cows six to eleven times per year. The farmer can order testing of *S.* Dublin antibody measurements in the same samples after they have been tested for production parameters. The individual milk ELISA is run very similar to the bulk tank milk test and has been evaluated against faecal culture results from cows collected on the same day as the milk sample. Two methods were used to evaluate the validity of the individual milk ELISA, the classical approach in which faecal culture is used as a gold standard test and the latent class approach in which estimates of sensitivity, specificity and prevalence in two population groups are obtained through a mathematical optimization procedure (Nielsen, 2003). At cut-off 25 ODC% the individual milk ELISA had an estimated sensitivity of 77% to 78% and an estimated specificity of 65% to 86% depending on which test evaluation method was used. At cut-off 50 ODC% these estimates were 42% to 43% and 81% to 94%, respectively (Fig 3.2).

In practice it does not make much sense to use a single individual ELISA test results to determine if an animal is infected now or is a carrier of *S.* Dublin. One needs to consider the dynamics of the antibodies in serum and milk upon new infections and repeated infections (Robertsson, 1984). The age of the animal also needs to be taken into account, because the validity of ELISAs has been shown to be best in young stock between three to ten months of age, slightly worse with regard to specificity in older animals and poor in calves below the age of three months (Nielsen and Ersbøll, 2004; Nielsen et al., 2004b). Figure 3.3 illustrates the typical antibody response measured in a cow that became infected three to four weeks after calving. Sampling was started at time of calving. Faecal shedding was noted for approximately one week. Seroconversion was measurable two weeks after the cow started shedding bacteria. The data comes from a field study in the Kongeå-project (Nielsen, 2003).

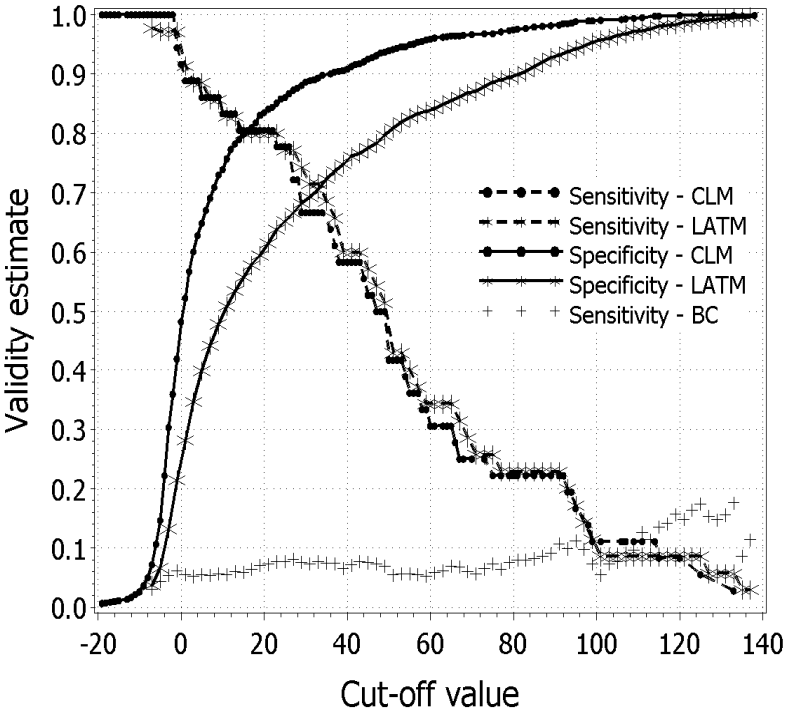

Figure 3.2 Validity estimates for faecal bacteriological culture (BC) and individual milk ELISA tests for detection of S. Dublin infected cattle calculated by classical test validation method (CLM) and maximum likelihood estimation which is a latent class test validation method (LATM) in two populations (Nielsen, 2003).

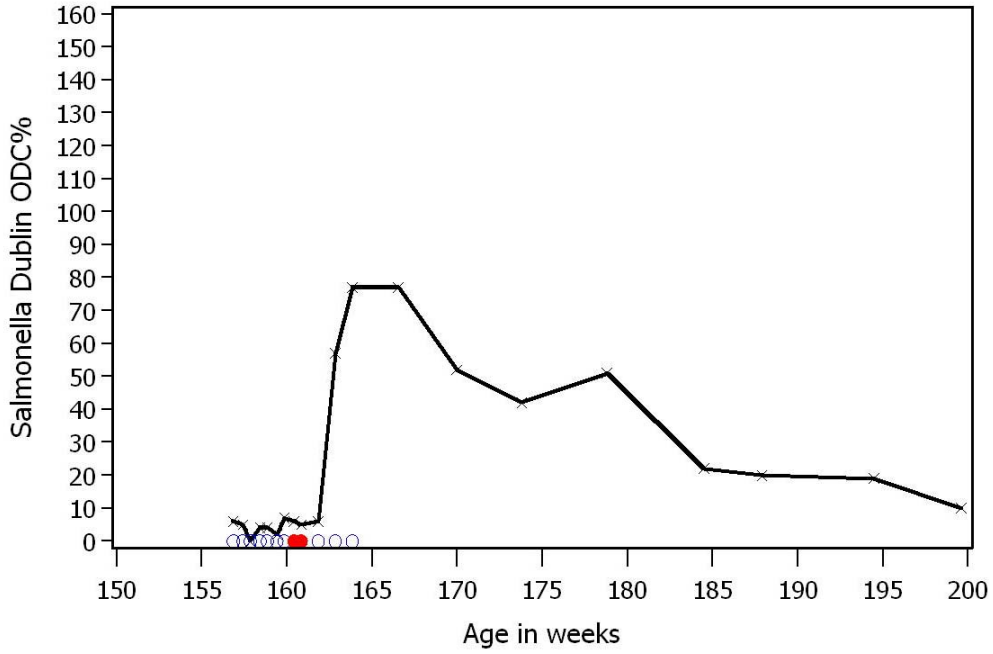

Fig 3.3 Typical course of faecal shedding (red dots) and antibody response measured by individual milk ELISA in a cow that became infected four weeks after calving, shed bacteria for approximately one week, seroconverted approximately two weeks after shedding of *S.* Dublin started and probably cleared the infection allowing antibodies to decline back to low levels (below 25 ODC%) within approximately 5 to 6 months.

One way that a single round of individual milk samples of all cows may be useful is if the farmer wants to know if the bulk tank milk is high in antibodies due to a few high titre cows or if it is a more general increase in individual milk that causes the bulk tank milk to go or remain high. For instance, towards the end of an intervention period the bulk tank milk may be kept high in antibody concentrations by a few older cows as exemplified in Figure 3.4a and 3.4b. Repeated individual milk samples with 3 to 4 months intervals can show which cows remain high in antibody titre and may have to be culled due to a risk that they are latent carriers that may re-infect the now susceptible herd. Even if the high titre cows are not all latent carriers it may be an advantage to cull them at the end of the intervention period to reduce the bulk tank milk antibody level which should ideally be close to 0 ODC%. This also removes the unknown risks associated with potential carriers' presence in the herd.

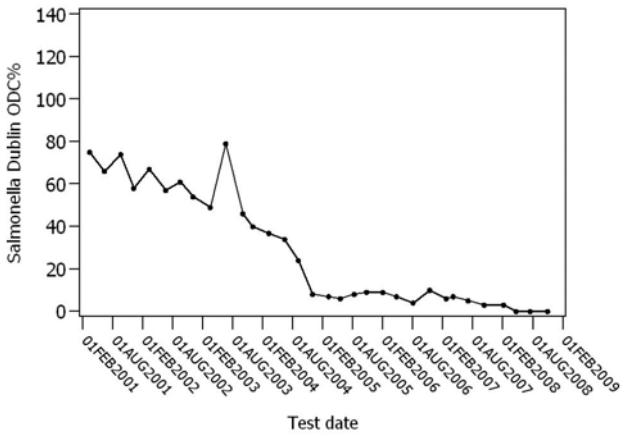

Fig 3.4a Bulk tank milk ELISA results from a herd under intervention against *S.* Dublin from 2003.

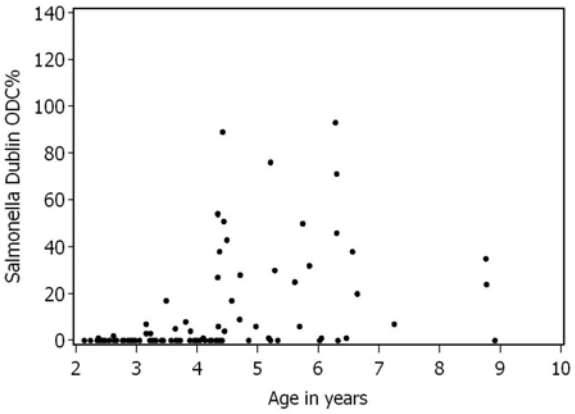

Fig 3.4b Individual milk ELISA results from cows in January 2004 from the same herd as in Fig 3.4a. Around this time the bulk tank milk ELISA response was 40 ODC%.

Serum ELISA

Antibody measurements in serum samples from young stock is a very useful tool in cattle herds both for herd diagnosis (Veling et al., 2002) and for evaluating progress in intervention. The serum ELISA that is used in Denmark has been evaluated in two studies (Nielsen and Ersbøll, 2004; Nielsen et al., 2004b). The sensitivity is similar to the individual milk ELISA. In the surveillance program a fairly high cut-off is used, 50 ODC%, which leads to an estimated sensitivity of 45% to 74% depending on the age of the animal. This sensitivity estimate denotes the probability that the test will be positive given that the animal is truly infected. Note that the sensitivity of the individual ELISAs is higher than for the faecal culture method, even at fairly high cut-offs. At cut-off 50 ODC%, the serum ELISA has an estimated specificity of 89% to 100% depending on the age of the animal and the estimation method used.

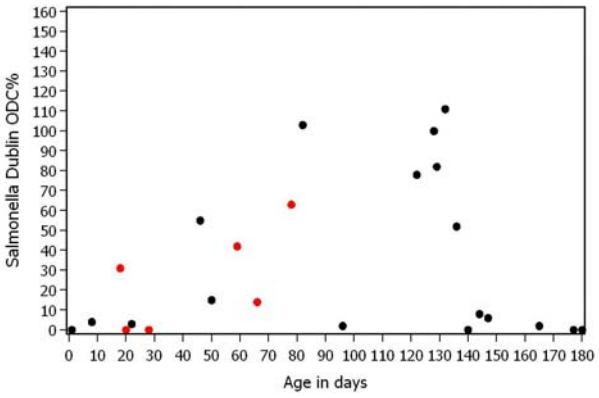

Fig 3.5a Antibody measurements from calves 0-180 days old in a herd with faecal shedding of *S.* Dublin detected in the same age group.
Faecal shedders are marked in red. Black dots indicate calves that were tested with faecal samples but were culture negative.

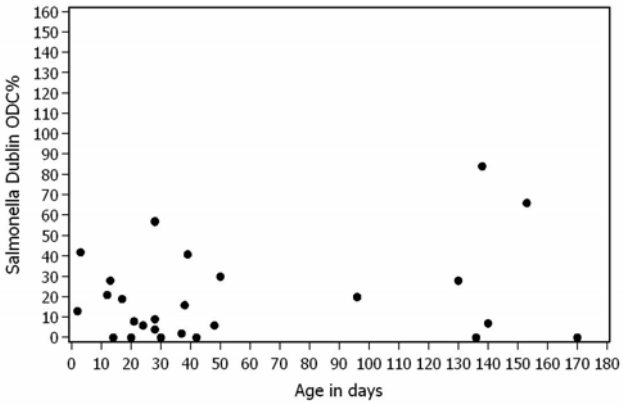

Fig 3.5b Antibody measurements from calves 0-180 days old in a herd where faecal shedding of *S.* Dublin was detected in the older age groups on the same day, but not in this age group.

Calves from three months to approximately six months of age is a good indicator group for testing, both because they are fairly easy to catch and sample, they are often indoors and the test has the best accuracy for this age group. Calves in this age group that have not met *Salmonella*-bacteria before in their lives will not have any specific antibodies to the infection and ELISA results are usually very close to zero. If infection has occurred in that group either around time of calving or within the first three to six months of life, usually one or more calves will have ELISA result above 50 ODC%. Figure 3.5a and 3.5b show a couple of examples from herds with known shedding among young calves or cows in the herd. Figure 3.6a and 3.6b show examples of herds that have managed to stop the infection among young calves and no other animals were shedding bacteria in the herd on the test day. All data come from the Kongeå-project. In that project calves of all ages were sampled and it should be noted in the graphs in Figure 3.6a and 3.6b that some calves can have specific antibodies from colostrum, but these have disappeared by three months of age.

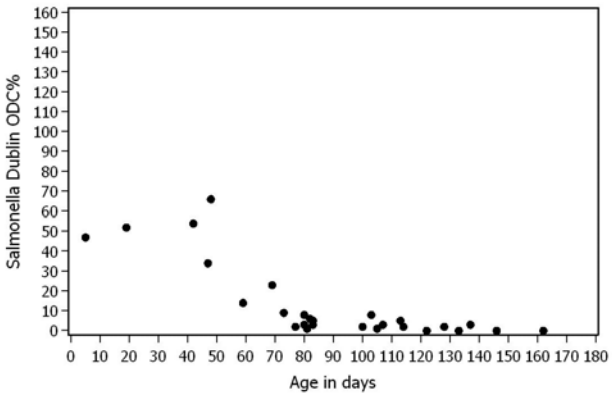

Fig 3.6a Antibody measurements from calves 0-180 days old in a herd with no faecal shedding of *S.* Dublin detected in the herd.

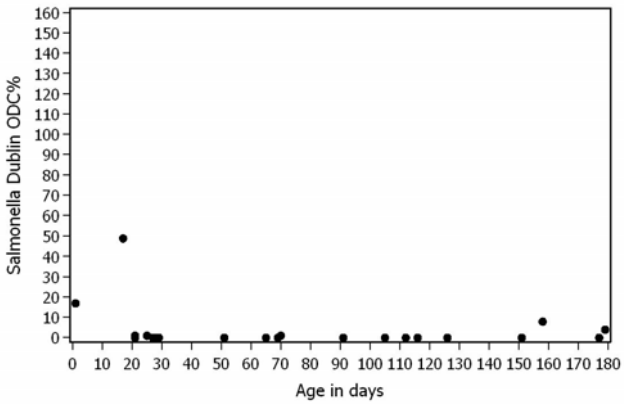

Fig 3.6b Antibody measurements from calves 0-180 days old in a herd with no faecal shedding of *S.* Dublin detected in the herd.

3.3 Interpretive summary of the diagnostic test section

This section gives an overview of the validity and test interpretation of currently available tests for detection of *S.* Dublin-infected cattle and cattle herds and for herd classification. Currently no individual, highly sensitive bacteria-detection test exists. Such tests would be useful for rapid and accurate outbreak diagnosis towards the end of the eradication campaign. They would also be useful for research purposes, in particular if it was possible to analyse many samples simultaneously at low costs. Real-time Polymerase Chain Reaction (PCR) methods may be a solution to improving the sensitivity of bacterial detection to some extend when serotyping is not essential, however specificity of this method has not been sufficiently documented. We are currently collecting field samples which will be used to establish the validity of a real-time PCR-test being developed at the National Food Institute.

I don't think we should be concerned that lack of highly sensitive tests will impede the eradication campaign, because the currently available diagnostic tests for antibody measurements in bulk tank milk and individual samples (ELISA) can be combined to obtain good sensitivity already a few weeks after the start of an outbreak of *S.* Dublin at reasonably low costs, if used correctly in the programmes. Moreover, we are currently trying to develop an early-warning system based on register-data from the Danish Cattle Database to improve early detection of *S.* Dublin outbreaks in dairy herds.

There are some specificity issues with these ELISA-tests both due to cross-reactions with other *Salmonella*-serotypes and because it takes time for the antibodies to disappear from a herd after *S.* Dublin-infection has been removed. This problem can be solved at herd level using individual testing and culling of high-titre cows towards the end of an intervention period. It will to some extend be annoying for the farmer, but will not reduce external biosecurity build into the national programmes today.

In practice it does not make much sense to use a single individual ELISA test results to determine if an animal is infected or a carrier of *S.* Dublin. One needs to consider the dynamics of the antibodies in serum and milk upon new infections and repeated infections. The age of the animal also needs to be taken into account, because we have shown that the validity of ELISAs is best in young stock between three to ten months of age, slightly worse with regard to specificity in older animals and poor in calves below the age of three months. However, antibody measurements in serum samples from groups of young stock are very useful in cattle herds both for herd diagnosis and for evaluating progress in intervention. Repeated antibody measurements at 3 to 4 month intervals can be used to group individual animals into risk groups to be handled separately at high risk times such as calving, or high-risk animals can be culled towards the end of an intervention period to avoid potential re-infection of the herd. This is discussed in more detail in the epidemiology section.

4. Epidemiology

Gathering information from the previous sections, empirical knowledge and experimental studies it is evident that calves below the age of 3 months are more susceptible to *S.* Dublin than older animals (Nazer and Osborne, 1977; Robertsson, 1984; Wray and Snoyenbos, 1985; Segall and Lindberg, 1991; McDonough et al., 1999; House and Smith, 2004; Silva et al., 2008) leading to more severe clinical signs and more shedding of bacteria in this age group. Thus, the incidence rate of acute infections is highest in young calves. However, both temporary and persistent infections can occur at any age if the infection dose is sufficiently high or if the exposed animals have reduced immunity, e.g. due to other diseases, starving, calving or stressful transportation (Chambers and Lysons, 1979; Mattila et al., 1988; Morisse and Cotte, 1994; Wray and Davies, 2000). A number of animals carry a latent infection, which may lead to periodical bacterial shedding mainly through faeces (Richardson and Watson, 1971; Richardson, 1973b; Wray et al., 1989). A small number of animals become supershedders i.e. carrier animals that shed large amounts of bacteria through faeces and in some cases milk more or less continuously for extended periods of time (House et al., 1993).

The previous sections have mainly dealt with animal level pathogenesis and infection dynamics, and how this knowledge can be used in diagnostic tests. This section provides a review of epidemiology of *S.* Dublin at herd level including prevalence, incidence and persistency, transmission routes and infection dynamics including within-herd prevalence changes over time and host-environment interactions of relevance for optimal control and eradication of *S.* Dublin in the field. Keep in mind that in this report, the term “eradication” means to reach a point where herd prevalence is reduced to close to zero with discontinued spread of infection between cattle herds.

4.1 Prevalence, incidence and persistency at herd level

Dairy herds

Since 2002, where the national surveillance program was started, prevalence of *S.* Dublin in cattle herds has been monitored through antibody measurements and herd classifications. In dairy herds, one way to follow the development in herd level prevalence is by plotting the proportion of Level 2 and 3 dairy herds over time. It immediately became evident that there were large variations in prevalence between regions of Denmark, so the country has been divided into some convenient regions that are followed over time as shown in Fig. 4.1 and Fig. 4.2. There was a clear drop in national prevalence from 26% to 16% over the first 3 years of the surveillance program. Since 2006, the national average has only reduced slightly to the current 14% whereas some regions, such as the southern part of Jutland (Jylland-Syd) and Himmerland, have had a dramatic reduction in Level 2 and 3 herds and other regions have remained quite stable in prevalence. The prevalence of dairy herds with too high antibodies and the prevalence of dairy herds not in Level 1 are monitored over time and the numbers are publicly available at www.kvaegvet.dk. One should keep in mind that these numbers are not equivalent to infected herds due to misclassification in the surveillance test scheme. Warnick et al. (2006) found that the test scheme validity in dairy herds varied with underlying true prevalence. Thus, for e.g. Himmerland where prevalence is high, the positive predictive value for Level 2 is higher than for low prevalence regions and at the same time, the negative predictive value is lower. In other words more dairy herds classified as Level 1 are in fact infected with *S.* Dublin in Himmerland than in the low prevalence regions. Yet, the monitoring provides a good indicator of the trend of the infection over time.

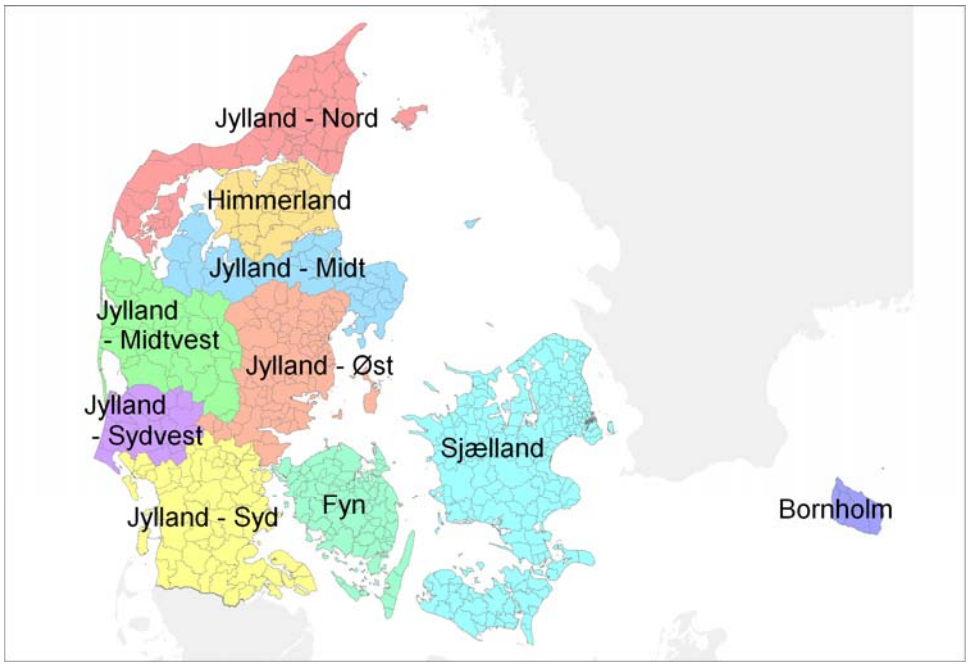

Figure 4.1 Map of regions used by the Danish Cattle Federation for *S.* Dublin prevalence monitoring in Danish dairy herds in 2009.

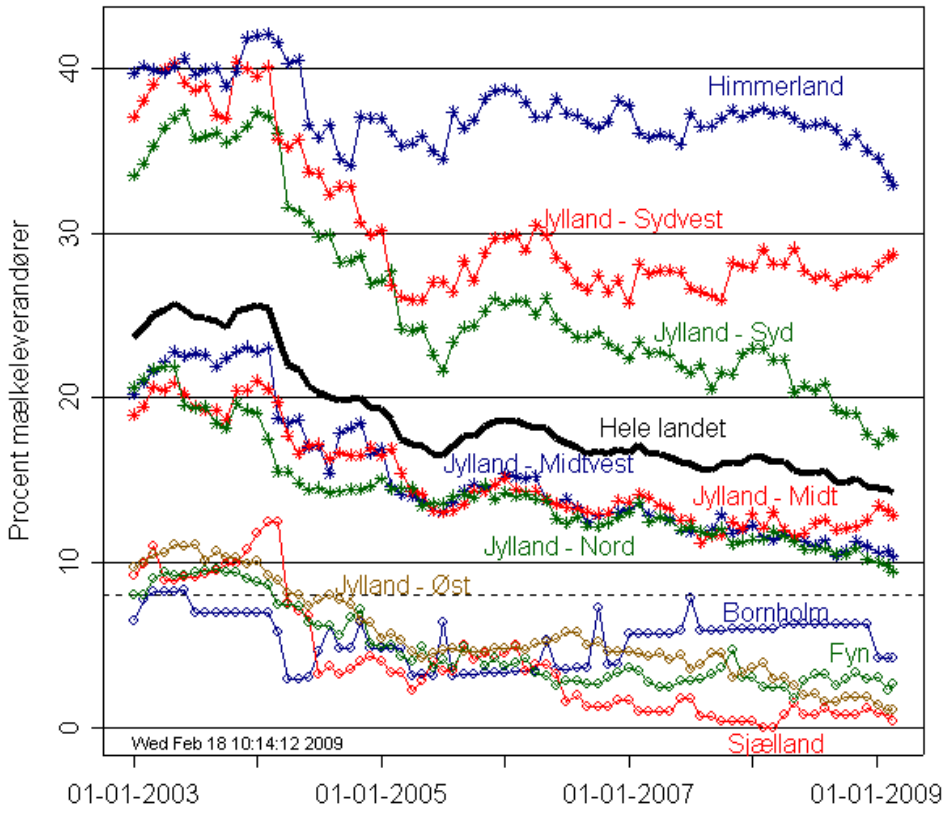

Figure 4.2 Regional changes in proportion of *S.* Dublin Level 2 and 3 dairy herds in Denmark from 2003 to 2009. The regions are displayed in Fig. 4.1.

A Danish study of incidence and recovery of *Salmonella* in cattle herds was based on register data from the surveillance program during the period 2001-2004. The first objective of that study was to evaluate risk factors for changing from Level 1 to Level 2 based on antibody measurements in bulk tank milk, which was indicative of herds becoming infected from one quarter of the year to the next. The second objective was to evaluate risk factors for changing from Level 2 to Level 1, which was indicative of herds recovering from infection between 2 consecutive quarters of the year (Nielsen et al., 2007a). The incidence of herd classification changes indicating new infection varied between 0.013 and 0.054 per herd-quarter of a year at risk. The highest incidences occurred in regions with high prevalence such as Himmerland. The incidence of herd classification changes indicating recovery varied between 0.093 and 0.207 per herd-quarter of a year at risk. The highest recovery rates occurred in regions with low prevalence such as the Islands (Fyn, Sjælland and Bornholm). In total, there were 421 more recovery events than incidence events during that time period. The factors that had the strongest association both in magnitude and significance with the probability of becoming infected in that study was region, local prevalence, purchase of animals from antibody positive herds and herd size (Nielsen et al., 2007a). Factors associated with highest probabilities of recovery were conventional (as opposed to organic) farming, smaller herd size, breed, no close-contact neighbours, low regional prevalence and few infected neighbours.

In another study based on the same type of register data, the duration of infection in *Salmonella*-infected dairy herds in Denmark during the first approximately four years of the surveillance program was evaluated to best be described by an exponential distribution with a mean of 726 days (almost 2 years) (Jordan et al., 2008). In a study from Sweden, the average duration of being under restrictions due to *Salmonella*-infections in cattle herds was around 200 days; however the authors mentioned that a few *S.* Dublin-infected herds were under restrictions for more than 600 days (Boqvist and Vagsholm, 2005). This should be seen in the light of extensive trade and control restrictions enforced by the Swedish *Salmonella*-program, which is based on elimination of the infection from all infected herds.

A study from the Netherlands suggests that approximately half of the dairy herds that experience an outbreak of *S.* Dublin become persistently infected and the probability that the infection becomes persistent in the herd depends on how well transmission can be limited early in the outbreak. Further, it was found that persistency could not be avoided solely by culling active carriers detected by faecal sample cultures (Veling, 2004).

Non-milk producing herds

Herd classification of non-milk producing herds is more complicated than for dairy herds, because it has to be based on either samples from individuals or pooled faecal samples. As described in section 3, faecal samples do not provide good sensitivity unless many samples or large amounts are collected, which then becomes very expensive. The cheapest method to obtain a herd diagnosis is to use ELISA measurements on serum samples. This is also the method that leads to the highest herd sensitivity if used in animals above 3 months of age. If for instance herd classification is based on eight blood samples, and each serum ELISA test has a (realistic) sensitivity of 74% and a specificity of 99%, the HSe= 63.9% and HSp=92.3%. The interpretation of this herd test is that the herd is *Salmonella*-positive if just one of the eight samples is above 50 ODC%. This example assumes a true within-herd prevalence of 15%. This example is similar to the herd classification system used today in the national surveillance system with some modifications for small herds. The samples are mostly collected at slaughter and as single samples over time, so the herd classification does not provide a good dynamic indicator of new infections and recovery of herd infections in the same way as bulk tank milk samples can. However, they do provide and overall indicator of prevalence in the non-milk

producing part of the cattle sector over time, and this is the main purpose of the test programme today. The numbers are publicly available at www.kvaegvet.dk. They show that approximately 1.2% of classifiable non-milk producing herds are seropositive today. However, more than 10% of the non-milk producing herds are not classifiable due to too few collected samples. These are mainly very small hobby herds.

The non-milk producing group of herds consists of a mix of many different types of herds including specialised dairy-beef slaughter calf production sites, heifer raising facilities, specialised beef production and breeding herds, dairy herds that recently ceased production, hobby herds and a mix of the different herd types. Therefore it is quite complex to get a good overview of how much *Salmonella* is present in these herds. A closer look at some defined categories of non-milk producing herds was performed in May 2008 for a report about the *S.* Dublin control programme in the Danish Veterinary and Food Administration. Table 4.1 shows the distribution of antibody positive herds in these groups. Note that a high proportion of the heifer raising facilities and "other" has too few samples to be classified. This is because many of these herds do not send animals to slaughter.

Table 4.1 Distribution of *S.* Dublin antibody positive herds in defined categories of non-milk producing herds in Denmark in May 2008.

Category (Category definition)	**Number of herds**	**Percent herds with *S.* Dublin antibody positive samples**	**Percent herds with unknown status due to too few samples**
Heifer raising facilities *(More than 30 animal years; more than 60% milking-breed and cross-breed heifers out of all animal years)*	420	7%	28%
Medium sized dairy-beef slaughter calf production sites *(Delivers 20 to 100 bull calves to slaughter per year; more than 80% milking-breed and cross-breed animals out of all animal years)*	553	17%	5%
Large dairy-beef slaughter calf production sites *(Delivers more than 100 bull calves to slaughter per year; more than 80% milking-breed and cross-breed animals out of all animal years)*	380	38%	1%
Medium sized beef breed herds *(Between 10 and 50 animal years; a minimum of 20% cows; beef breeds and cross-breeds constitute a minimum of 80% of all animal years)*	4.832	2%	6%
Large beef breed herds *(More than 50 animal years; a minimum of 20% cows; beef breeds and cross-breeds constitute a minimum of 80% of all animal years)*	1.046	2%	2%
Other *(Herds that do not fit into the above categories)*	9.943	5%	27%
Total	17.174	5%	19%

4.2 Transmission pathways

Transmission pathways for *S.* Dublin are mostly related to movement of faecal matter. They can conveniently be divided in two groups: between-herd and within-herd transmission pathways. Between-herd transmission of the pathogen is important for external biosecurity of individual herds and therefore must be blocked to avoid introduction of *S.* Dublin into a herd. Within-herd transmission pathways are important for internal biosecurity controlling spread of infection between animals, and between environment and animals. Within-herd transmission pathways affect the infection dynamics of the pathogen which again determine clinical expression, health reduction and economical impact in individual herds regardless of whether the infection is endemic in the herd or newly introduced. Transmission pathways within uninfected herds are only important once *S.* Dublin-bacteria become introduced to the herd. Therefore prevention of introduction of *S.* Dublin plays a major role in these herds. Once *S.* Dublin is introduced to a herd, the speed of dissemination of the pathogen is strongly dependent on management and structure of the barn sections.

Between-herd transmission

Introduction of *S.* Dublin to herds have been examined by several risk factor studies and by studying the possibility for transmission of the bacteria via vectors and infected cattle. The more closed the herd the lower the risk of introduction of the infection (van Schaik et al., 2002; Nielsen et al., 2007a). Purchase of infected cattle is known to be the most important cause of introduction of bacteria (Vaessen et al., 1998). This may be either by direct transfer from an infected herd or by infection acquired in transit, at markets or through dealers (Wray et al., 1990; Wray et al., 1991). The risk of introduction of *Salmonella*-bacteria has been shown to be four times higher when cattle was brought via markets or dealers than if transported directly between herds of unknown infection status (Evans and Davies, 1996). Carriers pose an important risk when moved from their original herd, because the stress of transportation and change of environment, feed etc. may lead to faecal shedding of high numbers of bacteria with no concurrent symptoms. Sharing of pastures or contamination of pastures by slurry from infected herds can be an important risk factor for introduction and disease outbreaks with *S.* Dublin in cattle herds (Taylor and Burrows, 1971b; Munch et al., 1987). However, some studies were not able to find an association between pasture contamination or sharing of grazing areas and increased risk of infection in cattle herds (Taylor, 1973; Vaessen et al., 1998), so the importance of this risk factor is unclear.

Several studies based on Danish surveillance data have shown that local spread is likely to occur between neighbouring herds. In one study there was a clear association between number of test-positive neighbour herds within a 2-km radius and the risk of changing to test-positive status while taking regional prevalence, herd size and purchase patterns into account (Nielsen et al., 2007a). In another study, it was found that the range of influence of herd test-status was between 1.5 km and 8.3 km (Ersbøll and Nielsen, 2008). The actual means of transmission between neighbouring herds are rarely found, so they remain speculative. Obviously, spread of slurry from infected herds on pastures is often under suspicion (Taylor and Burrows, 1971a; Findlay, 1972; Langvad et al., 2006). Transmission between herds may also occur with vectors or machinery. Vectors are hosts that can carry the bacteria either in faeces or externally on boots, clothing, tools, fur etc. Such vectors may be humans, cats, dogs, rats, birds and wild life. According to Gibson (1965) rats and mice do not play a major role in the spread of *S.* Dublin compared to other types of *Salmonella*-bacteria such as *Salmonella* Typhimurium DT104 (Davies and Wray, 1997; Davies, 1997). In one study it was possible to isolate *Salmonella*-bacteria from contaminated rubber boots 48 hours after casual rinsing (Kirk et al., 2002). Professionals that are in contact with many herds and many animals during the day pose the highest risk, and veterinarians and inseminators have been suggested as vectors of *S.* Dublin (Williams, 1980; Brooks, 1980).

Within-herd transmission

Within-herd transmission of *S.* Dublin vary considerably between herds and depends on structure and separation of different barn sections, stocking density, group sizes and compositions, movement of animals through the herd, and management related to hygiene and calving procedures. In one study, transmission between individually housed calves was shown to primarily occur via passive horizontal transfer between pens on utensils and barn equipment contaminated with infected faeces (Hardman et al., 1991). Splashing of a few drops of highly *Salmonella*-contaminated material into for instance feeding or water buckets can infect a new susceptible host (Bemis et al., 2007). *S.* Dublin can survive for several months in slurry (Findlay, 1972) and for years in dried faecal matter (Plym-Forshell and Ekesbo, 1996). Single housing is therefore recommended for young calves followed by all-in-all-out systems with thorough cleaning and disinfection between batches of calves kept in groups.

Carriers are probably important for infection persistency within-herd. Transmission from adult carriers to calves has been described (Richardson, 1973b). This is important around the time of calving when contact between carrier and calf is most intense. In this situation the carriers are subject to stress, which may lead to reactivation of latent infection or increased excretion of bacteria from carriers (Spier et al., 1991; Kehrli et al., 1999). Also, calves are highly susceptible just after birth (Fisher et al., 1976; Barrington and Parish, 2001). Carriers do not only pose a risk to their own calves in the calving environment. If no measures are taken to avoid cross contamination to the next calving cows and their calves, these may also become infected. Furthermore, excretion from carriers contaminates environment both indoors in barns and on pasture. However, even without carriers infection can remain persistent in a herd as long as there is enough entry of susceptible animals and/or sufficient environmental contamination from acutely infected animals (Nielsen et al., 2007b). Infection is also possible via aerosols (Wathes et al., 1988). Therefore high pressure cleaning of buildings and pens is a high risk procedure, if live animals are present. The buildings should be allowed to dry well before new susceptible animals enter the area.

4.3 Infection dynamics

Seasonality

All *Salmonella*-infections have a seasonal behaviour. Late summer and early fall is time for most incidences of infections in cattle and this is also when *S.* Dublin outbreaks peak almost every year. A study of Danish outbreaks of *Salmonella* in cattle herds from 1990 to 1998 that there was no seasonal difference between *Salmonella* Typhimurium and *S.* Dublin outbreaks in cattle herds. Further, the results of that study indicated that there is a positive association between temperatures from May to August and the number of isolations from clinical salmonellosis in cattle in Denmark (Steffensen and Blom, 1999). Fig. 4.3 illustrates the seasonal pattern in cattle herds that were under official veterinary supervision due to *S.* Dublin outbreaks per month from 2002 to 2009 in Denmark. There have been fewer recorded outbreaks over the years, but the seasonality is obvious almost every year with most outbreaks occurring in August to December. A seasonal pattern is also present in the serological measurements of bulk tank milk antibodies (Fig. 4.2) from the surveillance program though the trend is much less clear. This may be explained by the fact that it often takes some time after new spread of infection in a herd, before the bulk tank milk reacts sufficiently for herds to change status, in particular if the spread of infection starts in the calf barn.

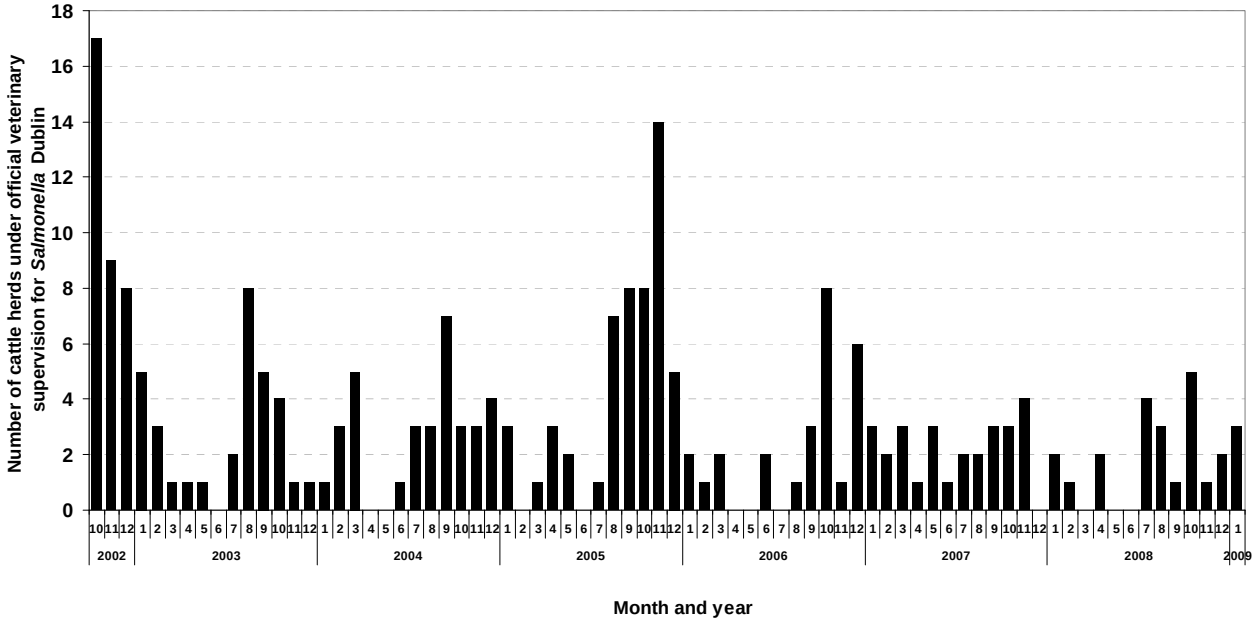

Figure 4.3 Number of cattle herds under official veterinary supervision due to salmonellosis caused by *S.* Dublin in Denmark from fall 2002 to 2009 (source: The Danish Cattle Database)

Within-herd infection dynamics

As described in the pathogenesis section (Section 2) in this report the development of infection in the individual animal is a dynamic process with different stages which cannot necessarily be followed easily during the course of infection with currently available diagnostic tests. We can improve our understanding of the process by measuring excreted bacteria and antibodies and by performing clinical examinations, but we will never be able to tell the true status or stage of infection in the animal for certain.

To study infection dynamics within a herd over time it is convenient to use compartments and pathways between the compartments with different characteristics that can be measured or at least estimated from repeated collection of data. This was done in a study by Nielsen et al. (2007b) based on field data from the Kongeå-project. Young calves were assigned to the categories susceptible, infectious, recovered based on faecal culture results and serum ELISA measurements. An environmental component was added to allow new infections to occur even when there were no calves classified as infectious present in the herd. This is most likely close to the real life situation in which bacteria can survive in the environment at lead to new infections next time susceptible animals are present in the barn area. In that study, the so-called basic reproduction ratio R_0 for *S.* Dublin was estimated in four endemically infected dairy herds. The basic reproduction ratio is the average number of secondary cases per week produced by one infected individual during the entire infectious period. R_0 varied from 1 to 2.5 in the four herds in that study. If R_0 is above 1 it is not likely that the infection will die out by chance and the infection typically becomes endemic. The infection is very dynamic in calf barns. As shown in Fig. 4.5 the number of calves in different compartments changes every week and calves are moved in and out of the barn area as part of the ordinary management of calves of different ages. Small groups of calves may eventually “run out” of susceptible calves unless new susceptible calves are introduced into the group continuously.

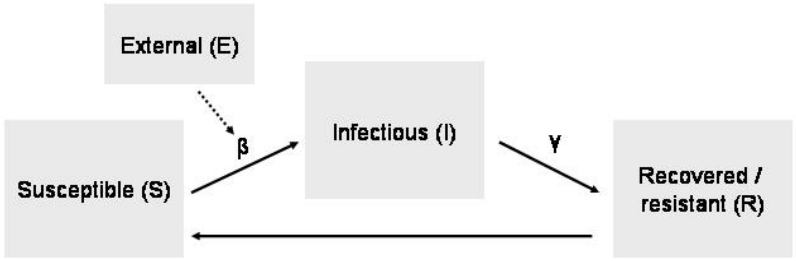

Figure 4.4 Compartments and pathways in a SIR-model used for estimation of the *S.* Dublin transmission parameter, β, in four endemically infected dairy herds. E is an external component that allows for new infections to occur due to environmental contamination and γ is the recovery rate (from Nielsen et al. (2007b)).

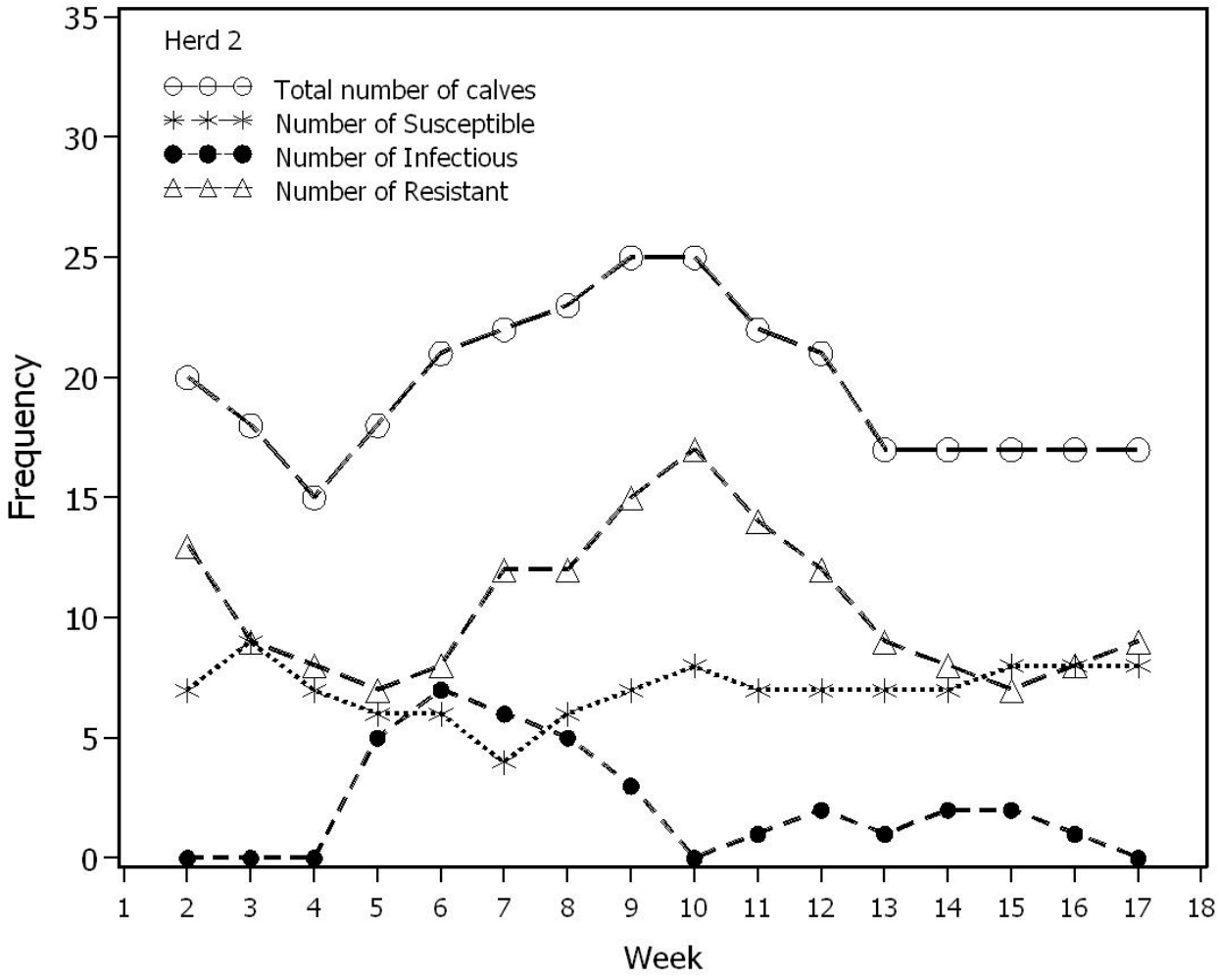

Figure 4.5 Fluctuations in the number of calves below the age of 180 days in different infection compartments over time in an endemically *S.* Dublin-infected dairy herd (from Nielsen et al. (2007b)). This herd experienced a small outbreak in the calf barn during weeks 5 to 9 of the study period.

In another sample collection that was part of the Kongeå-project, 29 dairy herds were visited four to five times at approximately 3 month intervals (Nielsen, 2003). At each visit all cattle present in the barns were sampled. From each animal a rectally collected faecal sample was cultured for detection of *Salmonella*-bacteria and all lactating cows had an individual milk sample collected. Non-lactating cows and young stock were sampled with blood samples for measurements of antibodies directed against *S.* Dublin LPS. Cut-offs were set to 50 ODC% in the ELISAs. Based on this sample collection, fluctuations in within-herd prevalence can be studied. Fig. 4.6a-d shows examples of result of i) apparent prevalence including all test results and ii) prevalence of faecal culture positive samples in three age groups from two different herds. Young stock is defined as cattle 180 days to 2 years-old.

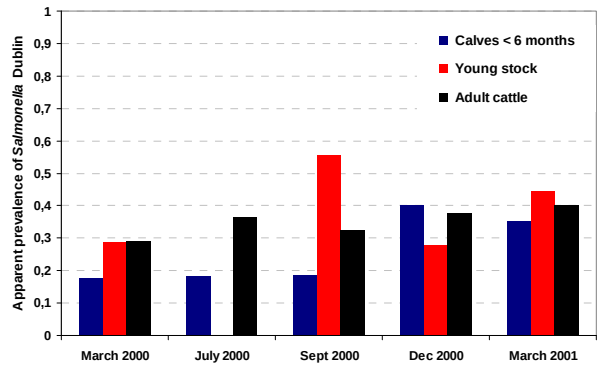

Figure 4.6a Apparent prevalence of *S.* Dublin based on serology and faecal cultures in a dairy herd.

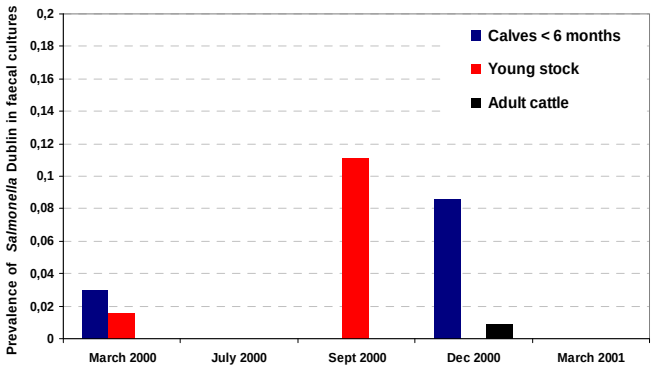

Figure 4.6b Prevalence of *S.* Dublin based on faecal cultures in the same dairy herd as in Fig. 4.6a.

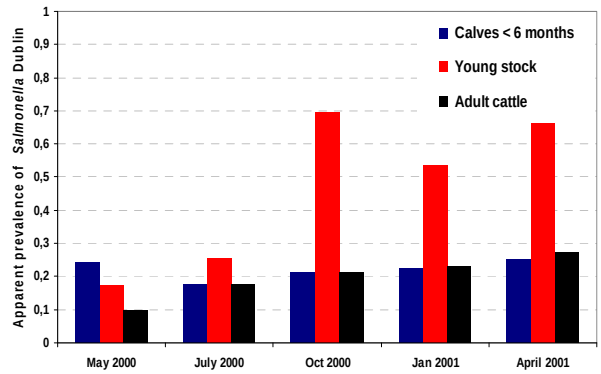

Figure 4.6c Apparent prevalence of *S.* Dublin based on serology and faecal cultures in a dairy herd.

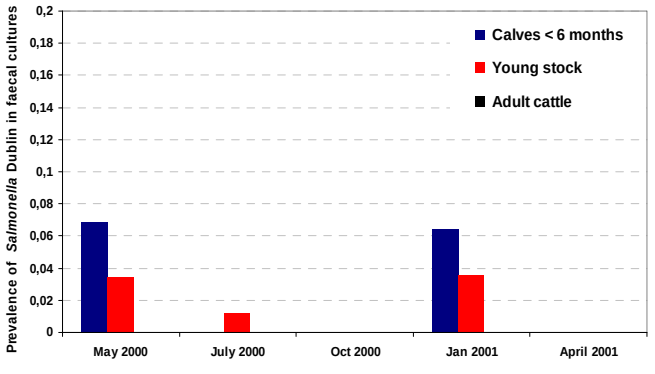

Figure 4.6d Prevalence of *S.* Dublin based on faecal cultures in the same dairy herd as in Fig. 4.6c.

The figures show that even though the apparent prevalence in each age group is fairly stable or sometimes quite high it does not necessarily reflect a high prevalence of shedders on the same day, e.g. in October 2000 in Figure 4.6c and 4.6d. Some of this may be due to low sensitivity of the faecal culture test. In Figure 4.6b the numbers behind the more than 10% shedders in young stock are 2 out of 18 tested animals.

Figure 4.7 shows the distribution of prevalences of faecal culture positive samples found in three age groups at 69 herd visits in 14 endemically *S.* Dublin-infected dairy herds.

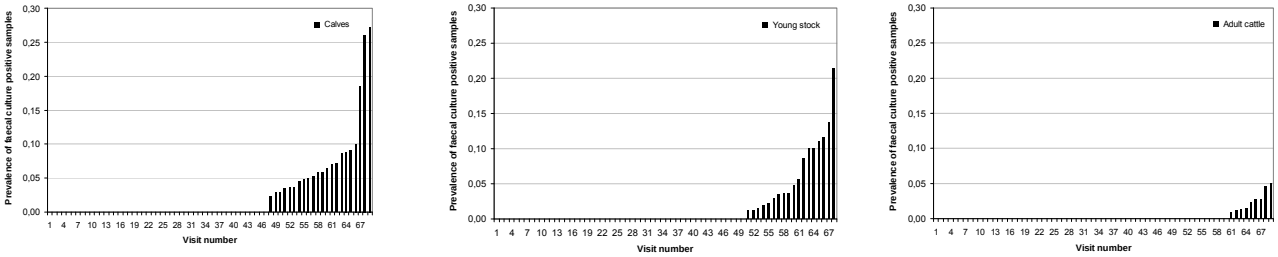

Figure 4.7 Prevalence of *S.* Dublin-faecal positive samples in calves, young stock and adult cattle in 14 endemically infected dairy herds that were visited 4-5 times each with 3 month intervals during 2000 to 2001.

An epidemiological study of repeated antibody measurements to detect carriers

It has been suggested that repeated antibody testing can be used to detect carrier animals to be culled as part of the intervention strategy in cattle herds (Smith et al., 1989; Spier et al., 1990; Smith et al., 1992). Based on the sampling activities described above I constructed a logistic regression model to estimate the probability of shedding at any given point in time for all ages of cattle and four antibody profiles.

ELISA results from animals below the age of 84 days (12 weeks) were not used, because the sensitivity and specificity of the test are known to be compromised by impaired capability of antibody production in calves below the age of 11-12 weeks (Da Roden et al., 1992) and maternally derived antibodies from colostrum (Nielsen, 2003). Based on practical experience with the test results and literature, ELISA results from animals from 84 days were used to group every individual into risk groups based on their serological profiles in the last up to four consecutive samples before the study period ended using the following criteria:

Risk group 1: This group was considered high risk of being a persistently infected carrier animal (R1).
Criteria: At least two samples available. The average of the last (up to) four samples and the most recent sample was above 80 ODC%. At least 120 days between the first, and the last sample above 80 ODC%.

Risk group 2: This group was considered to have moderate risk of being a persistently infected carrier animal (R2).
Criteria: The most recent sample above 50 ODC% or the average of the last up to four samples above 50 ODC% (but not above 80 ODC%).

Risk group 3: This group was considered very low risk of being a persistently infected carrier animal (R3).
Criteria: The average of the last up to four samples between 25-50 ODC% or the most recent sample between 25-50 ODC%.

Risk group 4: This group was considered low risk of being infected (R4).
Criteria: The average of the last up to four samples below 50 ODC% and the most recent sample below 25 ODC%.

Using these definitions, an animal that was only sampled once could only be grouped in Risk groups 2, 3 or 4 depending on whether that one ELISA measurement was above or below 25 and 50 ODC%. The age was recorded at the last sample. Having approximately three months between each sample date, the definition of the risk groups would be based on up to one year's worth of samples from the animals. However, many

animals did not have four samples collected, either because they entered or left the herd during the study period or because they were out on pasture during the summer period and could not be sampled. All animals above the age of seven months at the last sample event were included in the dataset for analysis. This was to avoid including animals that could not have been detected as carrier animals yet due to the 120-days-between-samples criteria. The distribution of cattle in the four risk groups is shown in Table 4.2. Fig. 4.8 illustrates the associations between age and risk groups and the probability of shedding of *S.* Dublin.

Table 4.2. Number and proportion of animals and faecal culture positive (FC-pos) animals in each of four risk groups based on serological profiles of all animals above the age of seven months in 14 dairy herds infected with *S.* Dublin.

Risk groups	**N in risk group (% of total)**	**FC-pos (% of N)**
R1: Persistently high serology (>80 ODC%)[a]	206 (7.7%)	7 (3.4%)
R2: Moderately high serology (50-80 ODC%)[a]	704 (26.4%)	26 (3.7%)
R3: Medium to low serology (25-50 ODC%)[b]	574 (21.5%)	6 (1.0%)
R4: Low serology (25 ODC%)[b]	1185 (44.4%)	7 (0.6%)
Total	2669 (100%)	46 (1.7%)

[a,b] Risk groups with statistically different probabilities of having *S.* Dublin-faecal culture positive samples

The conclusion from this study is that cattle above the age of 4 years have very low probability of shedding independent of the serological profile whereas younger cattle have significantly higher risk of shedding if the last sample is above 50 ODC% or high average antibody measurements in repeated samples than if the antibody level is below 50 ODC%. The probability of shedding is approximately 5 to 10 times higher in high risk serology profile groups R1 and R2 and low risk profile groups R3 and R4 among young stock. Overall, the highest estimated probability of shedding is around 11%. The true probability is probably 3 to 4 times higher than that due to low sensitivity in the faecal culture method used for this study.

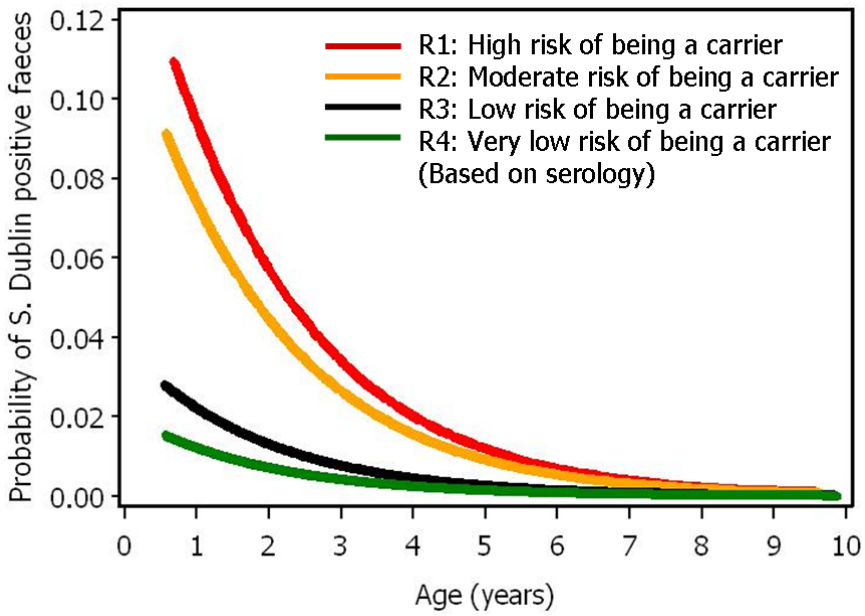

Figure 4.8 Model predicted probability of finding at least one *S.* Dublin-positive faecal culture when sampling every three months over a period of one year vs. age of the animals in four different risk groups based on serological profiles.

Herd level prevalence changes over time under different control scenarios

In a study performed at the International EpiLab in 2006 to 2007, register data and knowledge from epidemiological studies and field data were combined in a simulation model to evaluate the effect of different control scenarios for *S.* Dublin on a national level over a 10-year period (Jordan et al., 2008). The model was constructed as a 'virtual hierarchy' model which arranged animals, herds, and geographic regions in the Danish cattle industry as a hierarchy of objects in computer memory. Superimposed on all objects were an infection–recovery cycle, a control programme, and surveillance based on test results and animal movement. The infection-recovery cycle included a true negative period, dissemination period, antibody lag period, true positive period and antibody fall period that each herd would go through upon becoming infected to represent susceptible, infected, infectious and recovered periods for the herds. Distributions determined the different periods to represent variability in the infection cycle. All 7000 dairy herds in seven regions of Denmark were assigned a true infection status and a surveillance status in each daily time step of the simulations. Simulations consisted of multiple iterations to represent the stochasticity of the process. A long list of assumptions regarding within-herd prevalence, infectiousness of individual animals, within-herd dissemination of infection, recovery, etc. was used to mimic elements of importance for spread of infection between herds. An external environmental probability component (EEP) was added to allow for new infections to occur without movement of cattle between herds. Herds were assigned one of three purchase policies based on distributions estimated from data from the Danish Cattle Database (closed, conservative and indiscriminate purchase policies). Several control scenarios were tested in the model. These scenarios and the results of the model simulations are presented in Table 4.3.

The study suggested that enhanced internal and external biosecurity are preferable methods to reaching the goal of eradication of *S.* Dublin from the cattle population by 2014. However, one issue that the model did not take into is the structural changes in the cattle industry. The number of dairy herds has decreased from around 7000 in 2002 to 4400 in 2009 and the number continues to decrease. The number of cows has not decreased equivalently and herd size is rapidly increasing with new and larger farms being built. Thus, it is unavoidable to move cattle between herds to a greater extend than suggested by scenario 3. The conclusion is that a combination of improved internal biosecurity in infected herds and external biosecurity in both infected and non-infected herds is necessary, maybe combined with an early warning system to detect newly infected herds faster than today is recommended incorporated in the national strategies.

Table 4.3 Control scenarios and results from a virtual model of *S.* Dublin in the Danish dairy cattle industry.

Scenario # and name	Short description of scenario	National median herd prevalence after 10 years
1 Base scenario	Approximates the current management of *S.* Dublin by the national surveillance program. EEP[§]=10^{-5} Herds were allowed to acquire replacement animals from any other herd regardless of region by only taking into account their simulated purchase policy. BTM* ELISA testing was performed at the usual 90-day interval.	3.25%
2 Regional restriction of animal movement	Herds seeking replacement animals could only acquire them from the herd's home region. This prevents high-prevalence regions from 'exporting' infection thereby protecting low-prevalence regions from external sources of *S.* Dublin infection. EEP=10^{-5} BTM ELISA testing was performed at the usual 90-day interval.	3.38%
3 Enhanced external biosecurity	Limited all herds to no more than 12 purchase events per year and the number of animals acquired at any one purchase to 12. The proportion of herds with an 'indiscriminate' purchasing policy and the proportion of herds with a 'conservative' purchasing policy were halved with the remaining proportion assigned a purchasing policy of 'closed'. EEP=10^{-5} and BTM ELISA testing was performed at the usual 90-day interval.	0.1%
4 More frequent herd testing	Herds were tested more frequently by reducing the interval between BTM ELISA tests to 30 days. EEP=10^{-5} and original purchasing policies.	1.55%
5 Enhanced control at herd level	Reduced the duration of time that individual herds spent in the true-positive period to half of the mean of the exponential distribution used to model the true-positive period in the base scenario 1. (In scenario 5 a mean of 338 days was used). EEP=10^{-5} and BTM ELISA testing was performed at the usual 90-day interval.	0.18%
6 Combination of scenario 2, 3, 4 and 5	Combined all the features of scenarios 2, 3, 4 and 5 to provide some indication of the maximum possible reduction in prevalence that might occur with this composite approach.	0%

*BTM= Bulk tank milk, [§]= External Environmental Probability for introduction of infection

(Source: Jordan et al., 2008.)

4.4 Interpretive summary of the epidemiology section

S. Dublin is currently fairly common in Danish cattle production herds even though prevalence has decreased over the last 6 years. Prevalence is highest in dairy herds that have 14 % antibody positive herds and specialised dairy-beef slaughter-calf production sites where prevalence varies between 17 % and 38 % depending on herd size group. There are large regional differences in prevalence, incidence and recovery rates. The duration of infection is on average two years in Denmark but varies a lot from herd to herd. Luckily, the duration of infection can be markedly reduced by management. Epidemiological studies and empirical knowledge from Denmark, Sweden and The Netherlands have shown what is required to reduce prevalence effectively.

Enhancing internal and external biosecurity are preferable methods to reaching the goal of eradication of *S.* Dublin from the cattle population by 2014. However, we need to take the structural changes in the cattle industry into account. The number of dairy herds has decreased from around 7000 in 2002 to 4400 in 2009 and the number continues to decrease. The number of cows has not decreased equivalently, and herd size is rapidly increasing. New and larger farms are being built. Thus, it is unavoidable to move cattle between herds. Thus, I recommend that the national strategy is based on a combination of improving internal biosecurity in infected herds and external biosecurity in both infected and non-infected herds, preferably combined with an early warning system to detect newly infected herds faster than today in order to shorten the duration of infection. Incentives to ensure that these improvements are performed in as many herds as possible are likely to be required.

Within-herd transmission of *S.* Dublin varies considerably between herds and depends on structure and separation of different barn sections, stocking density, group sizes and compositions, movement of animals through the herd, and management related to hygiene and calving procedures. Highly contaminated material such as fresh faeces from acutely infected animal can infect milk, feed, water, or animals directly in contact with the material via splashes or passive transfer on barn equipment or dirty clothing, hands, boots etc. Thus, intervention in infected herds needs to focus on blocking these transmission pathways with particular focus on avoiding spread of faecal matter to susceptible animals, through management. An essential problem is that this often requires changing of daily routines and in-grown habits by the farmers - a problem that is more likely to be a barrier for success in the eradication campaign than anything else.

Culling of carriers may be beneficial to avoid re-infection of the herd once management actions have been implemented successfully to stop spread of infection. However, due to the intermittent shedding patterns of most carrier animals it is not possible to accurately differentiate active carriers from non-shedding animals with persistently high serology. In some herds there will be many potential carriers on the culling list and it might not profitable to cull them all. Epidemiological studies of serological profiles paired with faecal culture tests suggest that heifers and first parity cows with at least two repeated measurements of antibodies above 80 ODC % at 4-month intervals have a risk of approximately 40 % to 50 % of shedding bacteria intermittently. Whether it is profitable and necessary to cull all animals with such serological profiles can best be studied by simulation modelling of specific herd scenarios where herd size, management practices, calf mortality, reproduction management, culling strategies etc. can be taken into account. It may very well be that recommendations that are good for one herd will not work or be too expensive in another herd. Today, farmers are often left with too general advice about culling strategies during *S.* Dublin-intervention. Therefore, a modelling tool for planning of optimal and herd-specific intervention strategies is highly desirable and we are currently developing such a model in a collaboration project.

5. Intervention at herd level

In spite of knowledge about performance of diagnostic test and transmission dynamics it is crucial for larger intervention programmes that the means of intervention have been tried in practice. The following describes results and experiences from a number of smaller intervention studies.

5.1 Dairy herds

In a 3½-year intervention-study of six persistently infected dairy herds with recurrent clinical cases amongst calves, a voluntary control program was based on changes in management, especially at calving and of the pre-weaned calves. Further, all adult cattle (>1 year) were monitored serologically and cows with persistently high *S.* Dublin-antibody titres (ELISA, OD>0.5) were recommended culled (Jensen et al., 2004). The success of intervention was evaluated clinically and by serology. All herds managed to control the infection and reduce the proportion of seropositive animals to very low. Also calf mortality was significantly reduced in these herds during the intervention period. However, one herd had a recurrence of clinical cases four years after the control effort was initiated and the authors concluded that whereas it is possible to control the infection, eradication is difficult to achieve. The study showed that several of the herds had trouble carrying through the recommendations for management changes and also purchased new animals from herds with unknown infection status. It could not be concluded if culling of persistently seropositive cattle was a significant contribution to discontinued transmission of bacteria in that study.

A recent two year Dutch case-control intervention study was designed to assess the effect of a test-and-cull procedure for control of *S.* Dublin in 50 infected dairy herds (unpublished, personal communication 2008, Maarten Weber, The Netherlands). The herds were divided into two groups, an intervention group of 21 herds and a control group of 29 herds. Only the intervention herds were provided with serological and bacteriological culture results and recommended to cull latent and active carriers. The study showed that culling of suspected carriers was effective in significantly reducing the seroprevalence and the time until the herd could obtain '*Salmonella*-unsuspected' status based on bulk tank milk sampling. However, culling of suspected carriers did not result in eradication of the infection, so management procedures still appear to be important for successful intervention against *S.* Dublin. Thus, eradication of *S.* Dublin in infected herds probably requires intervention to close major routes of transmission for an extended period of time (House and Smith, 2004).

Intervention trial in 11 Danish dairy herds

Intervention actions may vary from herd to herd dependent on the initial within-herd prevalence and the manager's view and willingness to spend resources on management procedures and testing-and-culling. Thus, the aim of the study presented here was to test an approach to eradicate *S.* Dublin from dairy herds based on a step-wise procedure provided in a manual for advisers and farmers (Nielsen and Nielsen, 2007). The stepwise procedure was designed to assure the necessary commitment of the farm manager and follow-up during the intervention period required to obtain daily and long-term intervention efforts:

1) risk scoring to detect open transmission routes within the herd
2) determining a plan of action
3) performing management changes to close important routes of infection
4) diagnostic testing to evaluate progress of intervention procedures
5) interpreting repeated testing of individual animals to detect high-risk animals for special hygienic management or culling
6) evaluation of the effect on within-herd prevalence in different age groups

Farmers were required to go through all steps in the procedure to participate in the study. To evaluate the effect of this approach we used serological testing, farmer interviews, group experience meetings and national surveillance program herd status to study the participating herds and gain practical experience with what is feasible and what appears to work and not work for successful intervention against *S.* Dublin. The study is currently under submission to an international peer-review journal.

All eleven herds managed to reach Level 1 in the national surveillance program, however three herds did not reach Level 1 in the study period from 2003-2006 during the next two years (Table 5.1). All herds performed some changes in management to try to stop transmission of bacteria. However, according to the farmer's own opinions there was much variation in both the number and consistency of intervention actions taken. The main intervention actions performed in each herds are listed in Table 5.2. On average it took 26 months (std. 18) to reach Level 1 in the national surveillance program classification from the date intervention actions were initiated (Table 5.2). One herd did not reach an apparent prevalence <5 % in young stock in the study period. Among those ten herds that did obtain an apparent prevalence in young stock <5 %, the mean time from initiation of intervention actions was 12 months (std: 13). Figure 5.1 illustrates bulk tank milk *S.* Dublin ODC% in a) eight herds that reached Level 1 before the end of the study and b) three herds that reached Level 1 after the end of the study period. Only one herd (Herd B) consistently culled cattle with persistently high antibody titres. Herds A, F, G, H and I culled some cows and heifers with persistently high antibody titres when convenient mainly towards the end of the intervention period.

Table 5.1 Overview of dairy herds that participated in a Danish *S.* Dublin-intervention trial in 2003-2006.

Herd	Date herd started in project*	Date Level 1 was obtained[$]	Apparent prevalence in young stock at first and last sampling round		Apparent prevalence in adult cows at first and last sampling round		Herd size (#cows) at start and end of study period		Breed
	Month year	Month year	Start	End	Start	End	Start	End	
A	Feb. 2004	July 2004	0%	0%	32%	0%	60	302	Jersey
B	Oct. 2003	July 2004	1%	1%	10%	1%	97	170	Holstein-Friesean
C	Jan. 2004	Feb. 2006	1%	0%	27%	0%	112	157	Holstein-Friesean
D	Feb. 2004	Oct. 2004	4%	0%	6%	1%	65	74	Holstein-Friesean
E	Oct. 2003	Mar. 2005	6%	1%	13%	1%	88	91	Holstein-Friesean
F	Dec. 2003	May 2006	7%	0%	26%	1%	71	102	Jersey
G	Oct. 2003	Sep. 2005	9%	1%	30%	0%	98	120	Holstein-Friesean
H	Dec. 2003	July 2006	31%	3%	25%	5%	189	201	Holstein-Friesean
I	Oct. 2003	Feb. 2008	9%	23 %	40%	4%	88	116	Holstein-Friesean
J	Dec. 2003	Nov. 2007	21%	3%	20%	8%	96	136	Holstein-Friesean
K	Dec. 2003	Oct. 2008	57%	5%	41%	21 %	68	67	Holstein-Friesean

* Defined as date of first individual milk sampling

[$] Level 1 indicates "most likely free from *S.* Dublin-infection" in the national surveillance program

Table 5.2 Actions, start dates and time to success for 11 Danish dairy herds in a *S.* Dublin-intervention trial

Herd	Start date of intervention actions[$]	Main intervention actions	# months from intervention started to young stock seroprevalence was below 5%[*]	# months from intervention started to Level 1 was obtained[¤]
A	January 2005	New barn build in 2004 followed by merging of three herds. One calving pen per calving, cleaned and disinfected after each calving, milk from high-risk cows not used. One high titre cow culled.	0	0
B	November 2003	Camera watch of calvings, fast removal of calves at high risk cow calvings. From September 2005 calves moved to outdoor calf hutches, heifers moved to heifer raising facilities off premises. Culling of persistently high titre cows.	0	8
C	January 2004	The first 1½ years of the intervention period calves were removed from the dam immediately after birth. No milk used to feed calves from high risk cows. Heifers moved to heifer raising facilities off premises.	0	25
D	January 2004	Calves were removed from the dam 1-7 hours after birth. Immediate removal of calf if cows appeared on high risk list.	0	9
E	January 2004	No milk fed to calves from high risk cows. Thorough cleaning of pre-weaning calf pens before introducing new calves. Hygiene in pre-weaned calf pens and feeding buckets improved.	0	14
F	January 2003	Outdoors calf hutches. Removal of calf from dam 1-7 hours after birth. Culling of persistently high titre cows, but not all persistently high titre heifers from January 2004.	15	41
G	January 2004	Single calving pens cleaned after every calving. Calf removed immediately after birth. Thorough cleaning of outdoor calf hutches before new calves introduced. Culling of high risk cows when convenient.	22	20
H	November 2004	Pre-weaning calf pens cleaned between each calf. Discontinued use of high-pressure cleaning indoors. All-in-all-out for all common calf section. Strict management of colostrum with bank of milk from low risk cows fed via a tube. Common calving area split into two – one for high risk cows and one for others from July 2005. Culling of persistently high titre animals in late stages of intervention.	24	20
I	October 2003	Calves removed immediately after birth. Pre-weaning calf pens cleaned between each calf. Persistently high titre cows culled if convenient.	N/A	52
J	July 2004	Heifer calves removed immediately after birth. Only milk replacement fed to calves.	28	41
K	May 2004	Calving moved to outdoors. Fast removal of calves at high risk cow calvings. Pre-weaned calves moved to outdoor calf hutches, however not consistently.	30	53

[$] Estimated from interviews with farmer
[*] Probably overestimated because blood samples were collected twice per year in the project period so seroprevalence could have been reduced to below 5 % at any time during the previous six months
[¤] Level 1 indicates “most likely free from *S.* Dublin-infection” in the Danish surveillance program

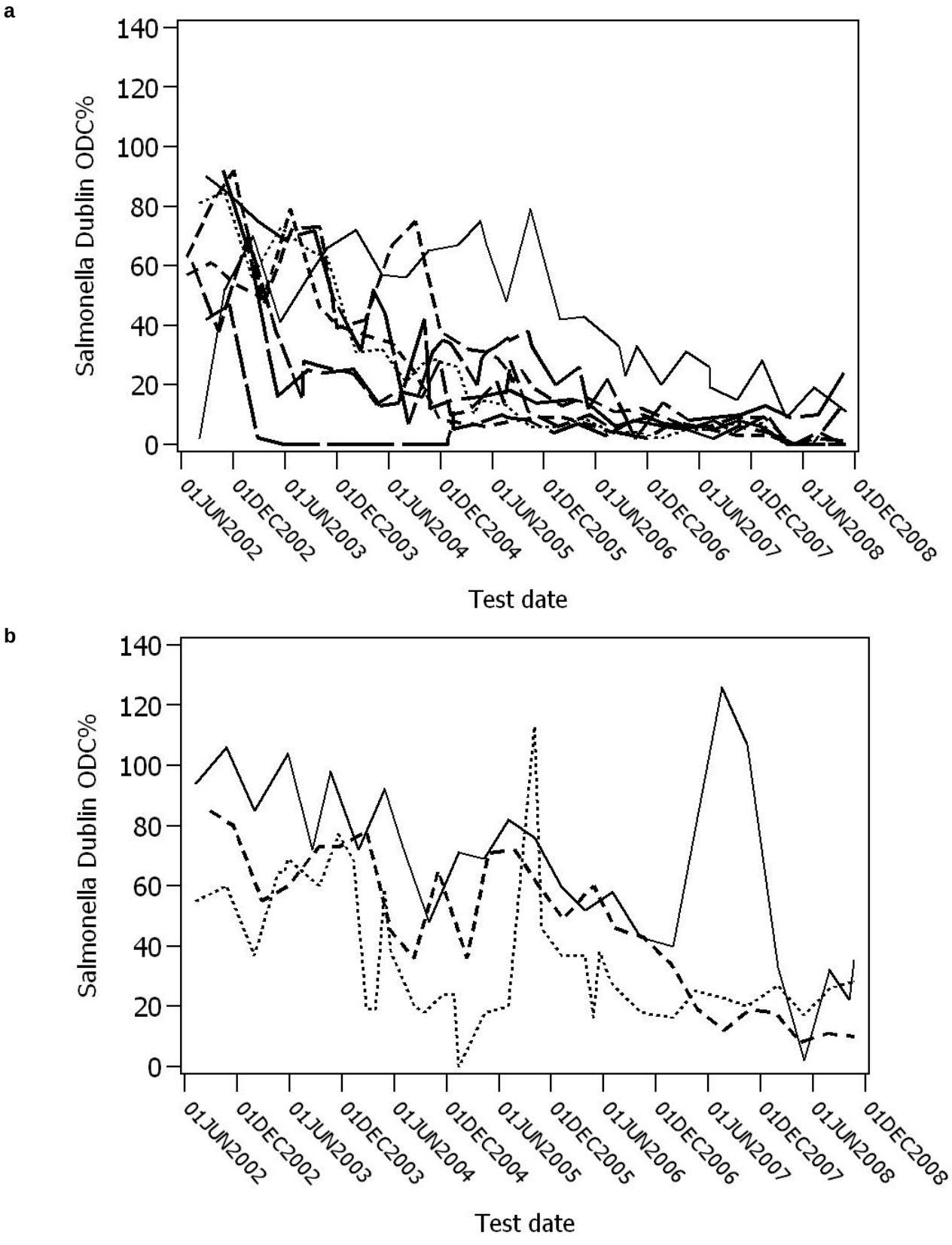

Figure 5.1 Repeated *S.* Dublin-antibody measurements (ODC%) in bulk tank milk from eleven intervention herds, a) eight herds that reached Level 1 in the national surveillance program before the study period ended and b) three herds that reached Level 1 after the study period ended.

Because young stock was only sampled twice per year it is difficult to exactly estimate when significant drops in seroprevalence occurred in young stock, however, it appeared that the drop in seroprevalence amongst young stock preceded a sufficient drop in bulk tank milk antibody measurements to reach Level 1 in most herds by 8-25 months. In Herds A, G and H reaching Level 1 in the national surveillance testing scheme and a young stock seroprevalence below 5% occurred more or less at the same time. In other words, the national surveillance classifications may not be the best indicator of successful intervention and more motivation tools were requested by the farmers. In this project, we used “progress graphs” based on individual antibody testing of young stock and lactating cows to demonstrate how well intervention actions were working in each herd. These progress graphs were sent out to the farmers every time new data was available i.e. 4-6 times per year with quarterly individual milk samples and biannual blood samplings. Figure 5.2 illustrates some of the progress graphs from Herd G as a representation of a herd with an unproblematic eradication course. Figure 5.3 illustrates progress graphs from Herd I as a representation of a herd with initial success followed by renewed transmission of infection. Both herds are in Level 1 today, but the time to get there has differed by 32 months.

During interviews with the participating farmers towards the end of the study period, all farmers and their local advisers expressed positive reception of these progress graphs as a tool to evaluate the success of the intervention actions performed in the herds. In particular, the young stock progress graphs are helpful because they can illustrate if intervention actions among young calves are working already after six months. Individual milk progress graphs are useful to show if high bulk tank milk measurements are caused by many or just a few cows in the herd, but sometimes also may illustrate if there is a high infection load in the calving pen in which case there will be many young cows with very high antibody levels in their milk. The progress graphs have since been implemented as tools that can be automatically generated from the Danish Cattle Database upon request from the farmer and pulled out directly to the herd or herd adviser via the cattle health management system “Dyreregistrering”.

5.2 Specialised dairy-beef production sites

Intervention in specialised dairy-beef production sites is challenged by the necessity to purchase young bull calves from dairy herds and bringing them together in groups often around the most susceptible time in their lives when maternally derived antibodies have weaned off. If just one of the source herds deliver *Salmonella*-infected calves it is difficult for the slaughter calf production site to avoid spread of the infection. Therefore, a project is currently running in Denmark in which it is attempted to eradicate *S.* Dublin from 20 slaughter calf production sites by a joint effort between the slaughter calf production herds and all dairy herds that deliver calves to these 20 herds. In the slaughter calf production herd the intervention recommendations are much the same as described for dairy herds in section 5.1 above. The recommendations mainly focus on strict all-in-all-out management procedures, smaller groups and generally improved hygiene. The dairy herds have to document that they only deliver calves free of *S.* Dublin. This is done by performing a risk assessment in the herd, making an intervention plan, performing intervention actions as described above and testing all calves present in the dairy herds at age 3 to 6 months with ELISA on blood samples to check that the intervention actions are working. The project will finish at end of 2009 and if successful this model will probably be used widely by slaughter calf production herds onwards. Preliminary results have shown that some slaughter calf herds in the project have already obtained solely antibody negative serum samples in calves aged 3 to 6 months after only half a year of intervention efforts. The advantage of this method is that it also increases the motivation by the dairy farmers to attempt to eradication *S.* Dublin from their premises if they can obtain a better market for their bull calves.

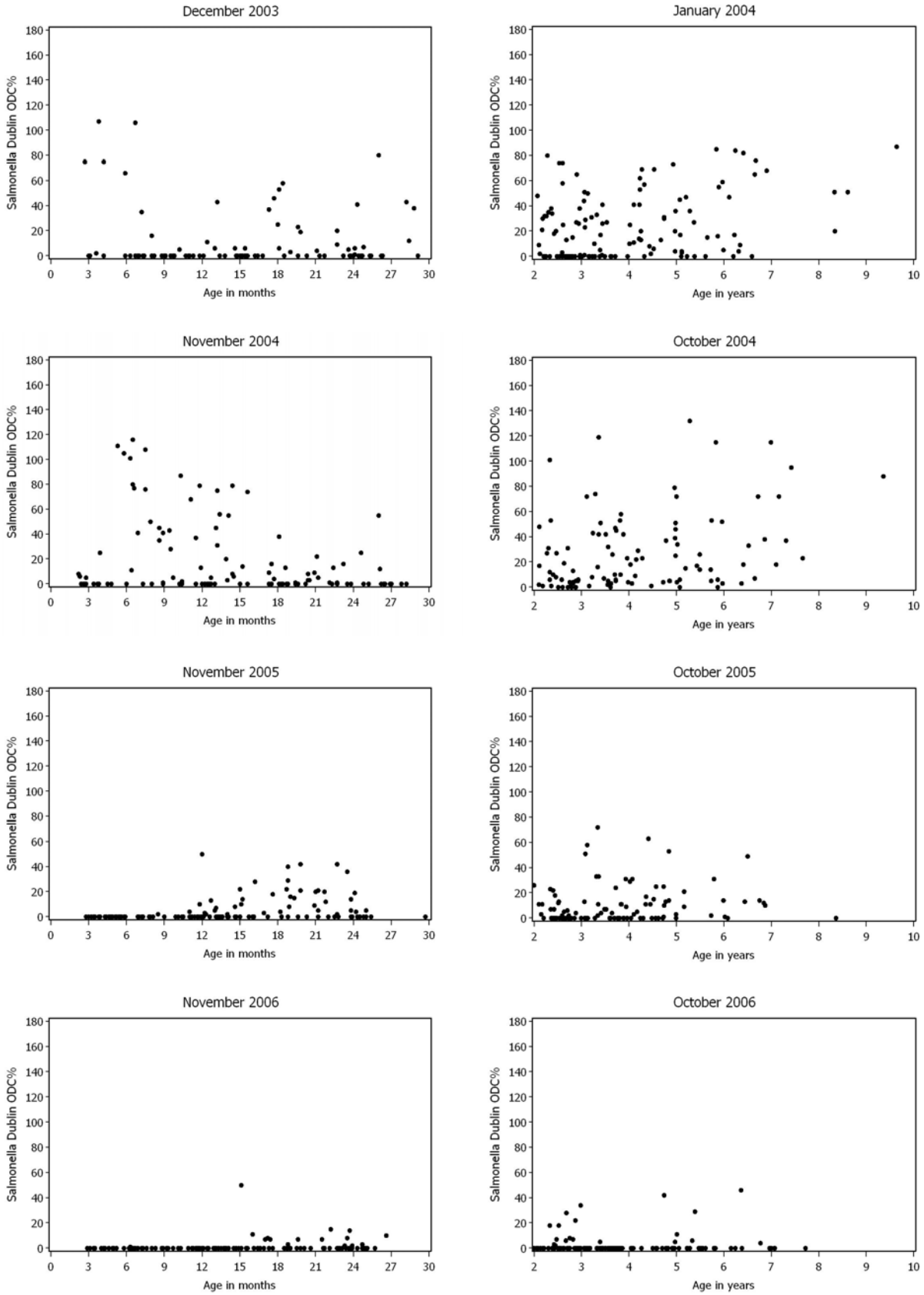

Figure 5.2 Progress graphs in a dairy herd (Herd G) in a *S.* Dublin intervention trial. Graphs to the left show antibody measurements in serum samples from young stock and graphs to the right show antibody measurements in individual milk samples from cows in 2003-2006.

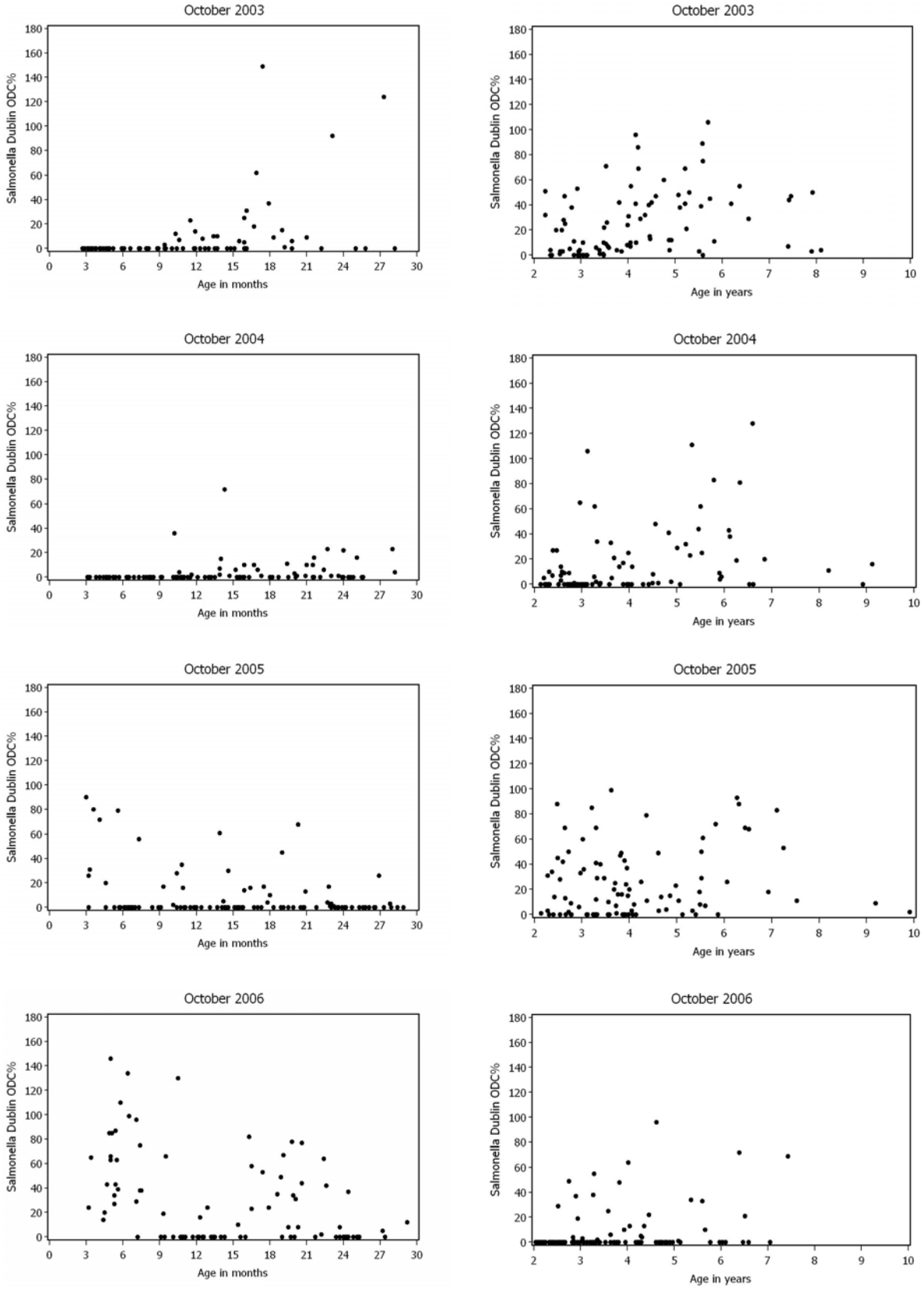

Figure 5.3 Progress graphs in a dairy herd (Herd I) in a *S.* Dublin-intervention trial. Graphs to the left show antibody measurements in serum samples from young stock and graphs to the right show antibody measurements in individual milk samples from cows in 2003-2006.

5.3 Preventive treatment of calves

S. Dublin serum

In Denmark, the company Dianova sells a product (Salmonella Dublin serum Vet.®) which consists of cattle serum with antibodies directed against *S.* Dublin O-antigens. It is given to young calves by intramuscular injection. It is meant for prevention of calves that are in risk of being exposed to *S.* Dublin-bacteria. The recommendation is to treat newborn calves and give two doses again 2 to 3 weeks later. This is fairly expensive for the farmer (120 DKK per dose), so the most common practice in Denmark is to only inject once just after birth in those herds that use it. Exactly how long this provides protection for the calf is unknown and published studies or other types of documentation of the effect of using *S.* Dublin-serum in infected herds are lacking.

S. Dublin vaccines

In some countries there have been efforts to develop vaccines against *S.* Dublin with varying success. In one experimental study of vaccine effect, calf mortality was significantly reduced in calves given a modified live, genetically altered *S.* Dublin-vaccine subcutaneously compared to a non-vaccinated control group (Selim et al., 1995). In another *study*, it was shown that giving a vaccine with an avirulent live *Salmonella choleraesuis* (strain 54) subcutaneously or intranasally to protect calves against salmonellosis caused by *S.* Dublin lead to significantly fewer clinical sign, less shedding and faster recovery of bacteria from organs (Fox et al., 1997).

Segall and Lindberg (1993) found that it was possible to improve immunity of calves around the age of 5 to 7 weeks using an oral live vaccine *S.* Dublin-strain as vaccine. The vaccine was given as three weekly increasing dosages of a so-called *S.* Dublin (O9, 12) hybrid strain SL7103 which only gave rise to mild, transient increases in temperature. Upon infection with high doses of a known virulent strain of *S.* Dublin (SVA47) calves exhibited only transient fever and mild mucoid diarrhea, which is a much milder course of infection than is seen in naïve calves. It did, however, not stop the infection from spreading to enterocytes of the jejunum and ileum, follicle-associated epithelium over the Peyer's patches and glandular tissues of the duodenal and tonsillar areas in the lungs.

In a study of a live oral *S.* Dublin-vaccine (genetically altered stable nonreverting aromatic-dependent (aro-) *S.* Dublin, strain SL5631) doses of 1.7×10^{10} given twice at age two and four weeks followed by infection with a virulent *S.* Dublin-strain (T2340). Protection was not evident and most calves (vaccinated or not) died upon the challenge (Smith et al., 1993).

An alternative approach was tried by Staak et al. (1989) who infused heat-inactivated *S.* Dublin-bacteria into the mammary gland of cows to protect their offsprings against salmonellosis via locally produced specific IgA and IgM in colostrum. The method provided some protection in that calves receiving colostrum from vaccinated cows, because they exhibited fewer clinical signs after challenge infection and had reduced excretion in quantity. However, the duration of excretion was similar to that of calves from unvaccinated dams.

The most successful vaccination trials to date was reported by Mizuno et al. (2008) who investigated safety, in vivo behaviour and protective properties of oral and intramuscular vaccination with live attenuated *S.* Dublin-mutant N-RM25. Vaccination by either route significantly reduced clinical signs and faecal shedding, prevented the development of systemic infection and protected calves from lethal challenge conducted within 14 days post-immunisation in calves below six weeks of age. Shedding of challenge bacteria was, however,

not fully prevented in any of the groups. The authors concluded that intramuscular administration of N-RM25 was safer than oral administration in terms of environmental contamination by the vaccine and provided better early onset protection in young calves.

Vaccination against *Salmonella* is not used in Denmark for several reasons. Probably a major reason is tradition, but also the fact that with live vaccines there is a concern about the environmental contamination. Although the bacteria used for the vaccines are usually reasonably harmless they are shed by the animals given the vaccines. Furthermore, the vaccination trials reported here are based on repeated treatments of calves. This is an approach that becomes non-profitable for the farmer in the long run. Vaccinations and serum treatments for protection are unlikely to lead to eradication of the infection, but may be useful in an outbreak situation where losses can be reduced until transmission of the infection can be reduced.

5.4 Interpretive summary of section about intervention

It is crucial for larger intervention programmes that the means and methods of intervention have been tried in practice.

Vaccination and other preventive treatments may be useful in outbreak situations to reduce production and animal health losses. However, excretion of bacteria is not fully stopped by vaccines, so it cannot be used to eradicate infection from herds. Another disadvantage of vaccines is the expense which makes it unprofitable to use vaccines for control of *S.* Dublin for prolonged periods of time in cattle herds.

Previous studies have shown that herds can control the infection and reduce the proportion of seropositive animals to very low through management, but culling of suspected carriers is not sufficient an alone-standing method to eradicate *S.* Dublin from infected herds. Moreover, farmers had trouble carrying through the recommendations for management changes and also purchased new animals from herds with unknown infection status during the intervention period. Thus, eradication of *S.* Dublin in infected herds requires intervention to close major routes of transmission for an extended period of time and tools are required to keep the herd manager focused and motivated and to avoid new infection from entering the herd from outside.

We have developed a manual based on herd-specific risk assessment for farmers and advisers to aid with the management planning. We have also developed tools for risk classification of individual animals based on repeated testing of individual animals. The manual is based on a step-wise procedure to ensure optimal intervention in each individual herd. This section describes a successful intervention trial in 11 dairy herds that used this method for intervention against *S.* Dublin. The method and tools have now been implemented as a central component in the national eradication campaign.

We are currently running a project to evaluate this methodology in specialised dairy-beef production sites. It is crucial to handle both risk at purchase and management of present infection in those types of herds.

6. National Strategies for *S.* Dublin control

In 1998, the Veterinary and Food Administration gathered a group of scientists from different institutions and the cattle industry to evaluate options for surveillance of *Salmonella* in cattle herds with particular focus on *S.* Dublin. This lead to two projects funded by the ministry and the Danish Cattle Federation that aimed at evaluating testing procedures for dairy and non-dairy herds.

In the dairy herd project it was found that there was a poor association between high antibody concentrations in bulk tank milk samples measured by a *Salmonella*-specific enzyme linked immunosorbent assay (ELISA) based of lipopolysaccharides (LPS) from *S.* Dublin and the probability of isolating *S.* Dublin-bacteria from faecal samples in the herd. At the same time, it was also found that herds with low antibody levels in bulk tank milk samples were very unlikely to be infected. In other words, the bulk tank milk test could be used in dairy herds to certify low antibody titre herds “low risk of *S.* Dublin-infection”, but the test could not be used to detect herds with high probability of being infected with the same accuracy (Pedersen.J.R., 2003). Thus, it was decided to base the surveillance program for dairy herds on three categorisations as described below. The program was later evaluated in an International EpiLab project. Results from this project are also given below.

The large group of cattle herds that do not produce milk in Denmark (currently almost 16,000 herds) is a very mixed group consisting of everything from small hobby herds to specialised dairy beef production sites and heifer raising facilities of all sizes, special breeding herds for beef cattle, herds with a mix of several production types and tradesmen. Therefore, designing a testing program for non-dairy herds is a challenge. The original testing program and the current program are described below. The scientific work that has been performed in this area concerns specialised dairy beef herds, because these are assumed to lead to the highest infection load at slaughter due to the prevalence and the number of animals delivered to slaughter. In one study, 2.3% of all tested slaughter calves (n=1703) from 34 large dairy beef herds were found *S.* Dublin-faecal culture positive at slaughter but the association between individual antibody status and excretion of *Salmonella*-bacteria at slaughter was poor (Anonymous, 2003). In total, 95% of all *Salmonella*-serotypes found in that study were *S.* Dublin. In a study from 2007-2008 of 71 dairy beef herds that delivered more than 100 steers to slaughter per year, *S.* Dublin was cultured from faeces in 1.5% of all tested slaughter calves (n=1296) (unpublished data). The current true prevalence of infected medium-sized and large dairy beef herds is around 25% on herd level roughly estimated from seroprevalence in the surveillance program (described below) and the above mentioned studies. The within-herd prevalence of shedders ranges from 1 to 20% in the slaughter age calves.

6.1 Surveillance for S. Dublin

Surveillance of dairy herds

The national surveillance program was initiated in October 2002. At that time all dairy herds were tested for antibodies directed against *S.* Dublin-LPS by an indirect ELISA on bulk tank milk (BTM) samples collected every three months (Nielsen and Ersbøll, 2005). The values obtained from the ELISA are called “ODC%-values” and can be considered semi-quantitative measures of the contents of antibodies in the samples expressed as a background corrected optic density compared to a positive control sample.

Herds were classified into one of three levels depending on BTM values, contact patterns to other herds and bacteriological culture results from samples submitted by local veterinarians as part of passive surveillance

of salmonellosis. Herds that had an average ODC% < 25 in the last four samples and that had not increased >20 ODC% in the last sample compared to the average of the previous three samples were classified ‘‘Level 1’’ which denotes ‘‘most likely free from *S.* Dublin-infection’’. Herds that did not stay below these cut-offs were classified ‘‘Level 2’’, which denotes ‘‘likely to be infected with *Salmonella”*, and herds that had been diagnosed *S.* Dublin infected based on bacteriological culture were classified ‘‘Level 3’’. These herds were often positive in faecal or organ samples submitted from herds with clinical problems. Herds in Level 3 were placed under restrictions by the veterinary authorities.
Trade or other types of contact (such as common grazing or contact via markets or shows) was included in the program by the use of the mandatory recordings of animal movements of individual cattle in the Danish Cattle Database. Contact to a herd in a poorer level would automatically lead to a lock in the same level for 3 months. For instance, if a Level 1-herd purchased cattle from a Level 2-herd it would be placed in Level 2 and locked in that level for 3 months. Level 2 was split into Level 2a which indicated Level 2 due to too high antibody levels in the BTM testing scheme and Level 2b due to contact to herds in Level 2. Likewise, Level 3 was split into Level 3a due to detection of bacteria and Level 3b due to contact to other Level 3 herds.

The locking system had a dramatic effect on the trading behaviour of Level 1 herds after the *S.* Dublin-levels became publicly available from October 2002 (Fig. 6.1). The scientific argument for keeping the locking system in place has been documented in a International EpiLab-project in 2004-5 in which it was shown that the risk of changing from Level 1 to Level 2 indicating new infection significantly ($p<0.0001$) and dramatically increases with the number of animals purchased from Level 2 herds in the previous quarter of the year (odds ratios varied from 3.8-11 depending on how many animals where purchased) (Nielsen et al., 2007). A similar effect was found for number of Level 2 herds purchased from.

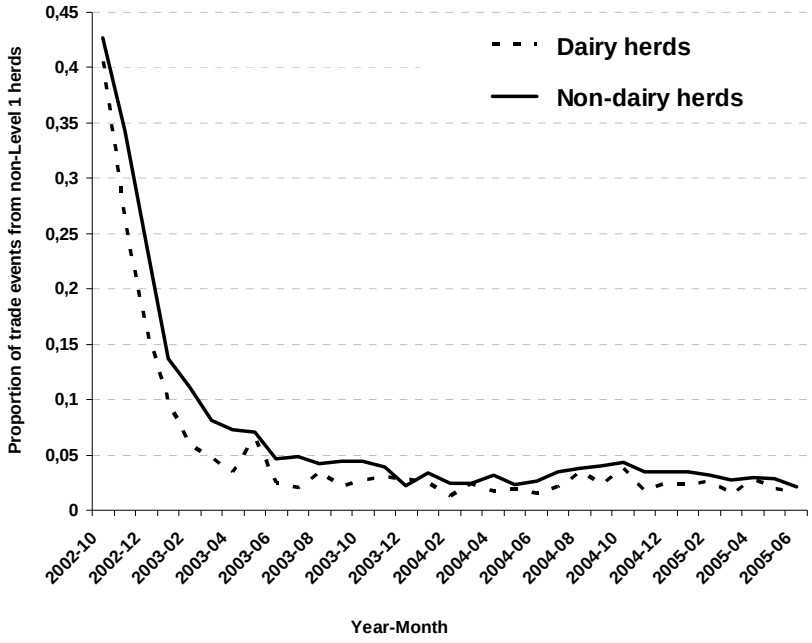

Fig. 6.1 Changes over time in the proportion of trade events performed by Level 1 dairy herds where the selling herd was not in Level 1 at the time of trade of cattle after the initiation of the surveillance program for *S.* Dublin in Denmark.

The program was changed slightly in 2006 to provide more incentives for the farmers to not only protect their herds against potentially incoming infection, but also try to actively try to control the infection in infected herds. Today, dairy herds can be placed in Level 1a based on the same criteria as described above for Level

1, or Level 1b which requires four consecutive BTM samples all < 40 ODC% and eight blood samples all < 50 ODC% from animals between 3-24 months of age. Herds that don't live up to these criteria are placed in Level 2 unless they have been diagnosed with clinical salmonellosis from *S.* Dublin, in which case they are placed in Level 3. Purchase from Level 2 or 3 will lead to a lock in Level 2 for a minimum of 3 weeks and after that the purchasing herd will have to retest the herd to be moved back to Level 1 (Anonymous, 2006a).

The changes in the percentage of BTM antibody positive herds (similar to Level 2) over time since the beginning of the testing programme in different regions of Denmark are illustrated in Figure 6.2.

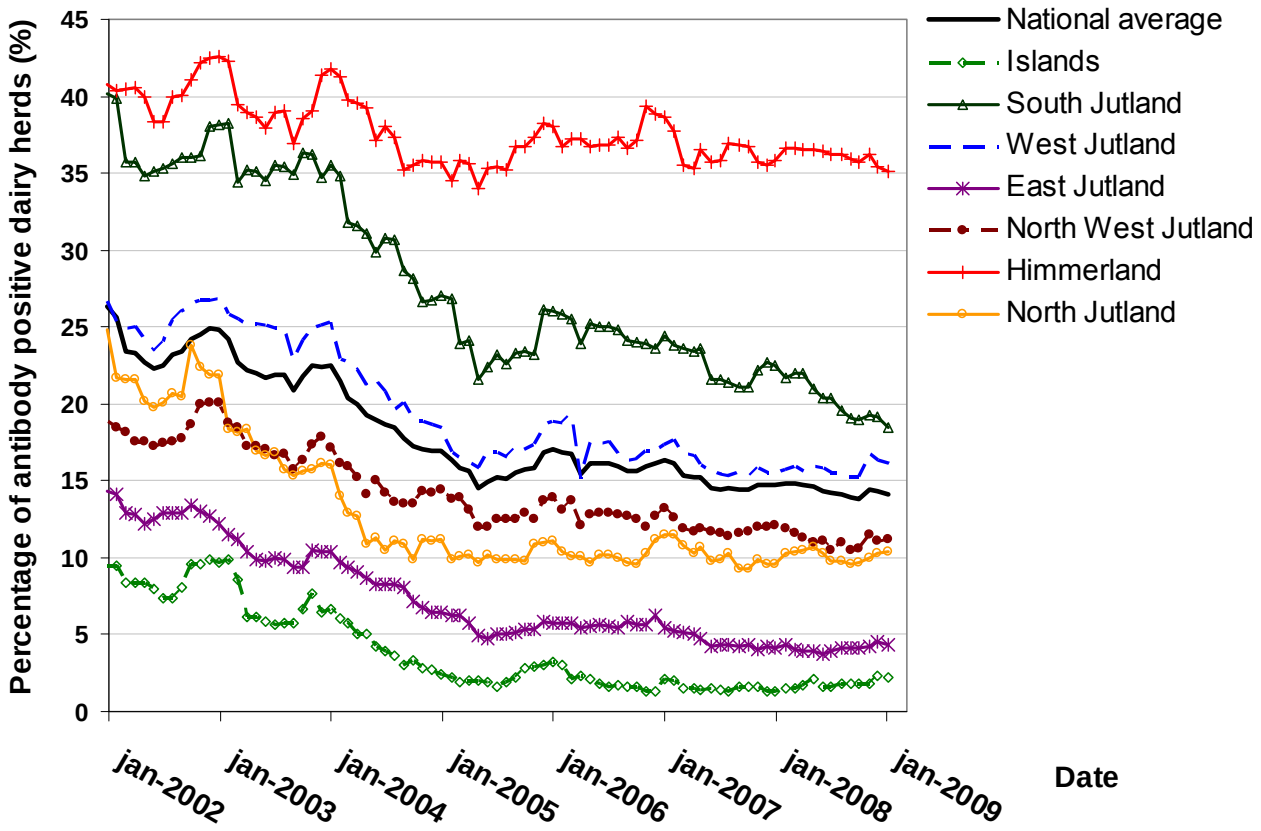

Fig. 6.2 Percentage of dairy herds that have been antibody positive in the bulk tank milk sampling scheme since the beginning of the national surveillance program for *S.* Dublin in different regions of Denmark.

On January 7, 2009, 14.1% of 4422 dairy herds were in Level 2 due to too high antibody levels.

In an International EpiLab-project guest scientist Dr. Lorin Warnick from Cornell University, USA, visited Copenhagen in 2004 and 2005 to perform an evaluation of the classification system. We performed the evaluation using a risk analysis model combining field data from previous field studies with a mathematical simulation model incorporating uncertainties and biological variation. It was estimated that 99% of Level 1 herds are truly non-infected and 80% (95CI: 68-84%) of Level 2 herds are truly infected at an overall true prevalence of 15% which is close to the current situation in Denmark (Warnick et al., 2006). The results also indicated that when prevalence becomes lower, the positive predictive value becomes lower which means that it may be necessary to allow for other ways to test the herd "most likely free from *S.* Dublin-infection". Based on these results approximately 500 dairy herds are still infected with *S.* Dublin today and correctly

placed in Level 2, 125 are not infected but are placed in Level 2 due to too high antibody levels in BTM. Approximately 3750 dairy herds are correctly classified in Level 1, and around 38 dairy herds are infected with *Salmonella* but their BTM-antibody measurements have not yet gone up to detectable levels in the current surveillance program. It is highly desired to reduce this number, because they may spread the infection unknowingly. We are currently testing a statistical model based on register data from all dairy herds to try to come up with a risk-based early-warning method of detecting the false negative herds in the surveillance program.

Surveillance of non-dairy herds

As mentioned before classification of non-dairy herds is complicated due to i) the structure of the population and ii) the diagnostic test performance at individual animal level. Clearly, the most accurate classification could be obtained by testing all individual animals but this is neither economically nor practically feasible. Thus, samples have to be taken from each herd. In practice, the easiest place to sample animals is at slaughter immediately after the animal is killed, and this is what is mainly used in the national surveillance programme for non-dairy herds today. However, not all herds send enough animals to slaughter to get a classification, so blood samples can also be collected in the herds.

Initially, the herd classification was based on three blood samples; however in 2006 it was changed so that non-dairy herds are now classified as follows:

Level 1b is obtained if the last eight blood samples from animals aged 3 months to 5 years are < 50 ODC% and it is more than 3 weeks ago that the herd was in Level 2. If the herd doesn't live up to these criteria the herd is placed in Level 2. Diagnosed clinical salmonellosis leads to Level 3 just like for dairy herds. Herds that do not have enough samples collected to be classified are placed in "unknown" level (Anonymous, 2006a). Antibody measurements do not outdate which means that over time there will be fewer and fewer herds in the unknown level. In January 2009, there were 1.1 % antibody positive non-dairy herds out of 15995 and 13.5 % further that could not be declared Level 1 either due to missing samples or contact to Level 2 or 3-herds. The same locks count for non-dairy as for dairy herds. Most of the approximately 160 herds in Level 2 are specialised dairy beef herds.

6.2 Eradication campaign

In 2006 an agreement was made between the Danish Cattle Federation and the Danish Veterinary and Food Administration that an eradication campaign was to be started from 2007 with the aim of eradicating *S.* Dublin from the Danish cattle population before the end of 2014. By eradication is meant that the prevalence has to be close to 0% and there should be no more than 5 newly infected herds per year and the infection in these herds must be effectively controlled so that it does not spread to other herds.

The ultimate purpose of this is that Denmark may be able to apply for special "*Salmonella*-free" status in EU and thus be able to ban import of *Salmonella*-infected meat from other countries (Anonymous, 2006). This involves all sectors of production animals and the different sectors are approaching it in different ways. The cattle industry has agreed to focus on *S.* Dublin and go through three phases:

Phase 1: Voluntary intervention in infected cattle herds from beginning of 2007 to end of 2009. In this period the surveillance program and the regulations regarding *S.* Dublin will not be changed. The Danish Cattle

Federation is running a large campaign to encourage farmers to start intervening against the infection in infected herds and make sure to protect their herds carefully in non-infected herds. This involves several research and intervention projects in the field. All Level 2 and 3 herds have been offered to be part of a network group that meets several times together with a consultant or veterinarian acting as facilitator to inspire each other to keep up the intervention work. Much of what needs to be done in a herd to eradicate *S.* Dublin is about daily management routines, so risk assessment tools have been developed to aid the farmers in figuring out the most optimal intervention strategy in his herd and test interpretation tools are also available, see the section about intervention below.

Phase 2: Price differentiation on milk and beef meat will be employed by the dairies and cattle abattoirs if it is found necessary to improve the motivation to eradicate *S.* Dublin from the herd. The surveillance programme may be adjusted if this is found necessary to reduce the transmission of infection from infected herds. This phase lasts from 2010-2012.

Phase 3: The Danish Veterinary and Food Administration may use new executive orders or changes to the current executive orders about *Salmonella* in cattle and swine to prevent infection spreading from *S.* Dublin-infected herds, for instance by closing the herd for trade of living animals. This phase lasts from 2013-2014.

6.3 Interpretive summary of section about national strategies

This section gives an overview of the two main national strategies for control of *S.* Dublin in Denmark.

A national surveillance program was initiated in 2002 and covers all cattle herds in Denmark. Continuous active surveillance is based on bulk tank milk antibody measurements in dairy herds and individual blood samples mostly collected at slaughter in non-milk producing herds. All herds are classified into three levels of infection risks and regulations to encourage trade with low-risk herds are in place. The program was successful in reducing the prevalence of seropositive dairy herds from approximately 26 % in 2002 to 16 % 4 years later. At that point the effect of the program on prevalence reduction weaned of and it was decided to start an eradication campaign from 2007.

The eradication campaign is split into three phases and we are currently in the last year of the first phase (2007-2009) in which the cattle industry attempts to reach 8 % antibody positive dairy herds through voluntary intervention efforts in infected herds. The campaign is based on massive information campaigns, projects with local network groups of farmers, research and intervention projects and direct contact to farmers and advisers through centrally hired veterinary consultants with expertise in *Salmonella*-management. From 2010 price differentiations will most likely be introduced on milk and beef to improve motivation to start intervention in infected herds. There is no doubt that some farmers are awaiting this phase before they start doing anything, but a lot of farmers have already started intervention in their herds, and the network-group set-up has been very successful underpinning the fact that local support of colleagues and advisers is essential for *S.* Dublin intervention.

I believe that we will be able to eradicate *S.* Dublin from the Danish cattle population, if we understand and accept that rules and regulations are not enough to reach the goal. Direct, human contact and interest in the farmer's individual herds and challenges are crucial. Veterinary advisers and cattle consultants could choose to see this as a profitable challenge, and local sector politicians should encourage and motivate to reach the goal throughout the process.

7. Perspectives

In this report, I have presented a mix of detailed state-of-the-art research, empirical knowledge and reasoning that I believe are fundamental to control and eradication of *S.* Dublin in the Danish cattle population. If we now move to a “helicopter view” on the process some preconditions stand out as essential to reach the goal of eradication (Jordan et al., 2008; Sandøe and Christiansen, 2008):

1. It must be possible to obtain reasonably accurate purpose related diagnoses on herd level to distinguish infectious or non-infectious animals and herds. Farmers and their advisers must know how to obtain such diagnoses and what to use them for.
2. It is important to block routes of transmission between animals and herds including those that go via contaminated environment in order to reduce the duration of infection in each infected herd to a minimum and prevent new cases.
3. A rigorous system for recording of animal movement, herd classification and monitoring system, official regulations and control guidelines must be available. This requires a central organisation of the national eradication effort and collaboration between all institutions involved.

Thanks to the abundant research into development and evaluation of diagnostic tests for herd level diagnosis of *S.* Dublin in cattle herds and thanks to a rigorous system for recording of animal movement etc. in the Danish Cattle Database, cattle herds are today tested and classified into surveillance levels so that farmers are able to obtain reasonable protection of their herds against infection when they need to purchase animals, if they choose to use the available systems. The fairly simple classification system was readily understood by the farmers at initiation of the surveillance program. Even if many didn’t like the idea about having to think about *S.* Dublin and external biosecurity, trade patterns were dramatically changed within 3 months after the program was started. Structural changes in the cattle sector have made it impossible to avoid movement of cattle from high-risk farms all together, but as prevalence continuously reduces this has and will become easier over time.

Denmark has the advantage of being small with all cattle owners organised within the same organisation, the Danish Cattle Federation, which has good collaboration and fairly direct contact to politicians and the Danish Veterinary and Food Administration in addition to having access to data from all herds in the Danish Cattle Database. All cattle in Denmark are ear-tagged within the first couple of days of life and all movements between herds are recorded. Movement of animals is already part of the surveillance program for *S.* Dublin, but it is likely that it can be used in more optimal ways to limit transmission of *S.* Dublin between herds in the future for instance by restricting movement out of infected herds or movement from high-prevalence regions into low-prevalence regions.

Thus, today point 2 in the above list of preconditions is the most challenging. The wide variations in the infection course and clinical expression of *S.* Dublin-infections in cattle make it challenging to communicate advice to farmers about how to control the infection. Often advisers and farmers seek simple advice similar to those used in the recent BVD-eradication campaign in which test-and-cull-procedures were central. As shown in this report, test-and-cull procedures are not the answer to *S.* Dublin eradication. Culling of suspected carrier animals can be helpful and speed up the intervention process in particular towards the end of the intervention period, but management actions to reduce the environmental infection load and prevent transmission of bacteria between animals are non-avoidable if eradication of the infection from the cattle population is the goal. In particular, good calving practices and good management practices of newborn and

young calves are essential. The advantage is that it also benefits the herd in relation other infections such as *E. coli* and paratuberculosis, and improves animal health and lowers calf mortality.

The key issue is to keep the farmer's motivation up for daily and continuous efforts to keep management in place to prevent transmission of bacteria and reduce contamination of the environment. These daily routines that often require manual work to clean barn equipment etc. are difficult to keep up in the long run in a farmer's busy daily schedule. We have experienced that small network groups that exchange experiences and keep each other focused on the challenge are very useful. Not all farmers want to join such groups so local advisers also have an important responsibility to support the process. But advisers need to be sharp, because recommendations that work for one herd will not work or be too expensive in another herd. Today, farmers are often left with too general advice about management and culling strategies during *S.* Dublin-intervention and they find it difficult to relate to. Research in the field of simulation modelling of herd-specific intervention scenarios in which the current knowledge about pathogenesis and test-interpretation can be incorporated is recommended. I believe that we will be able to eradicate *S.* Dublin from the Danish cattle population, if we understand and accept that rules and regulations are necessary but not enough to reach the goal due to human nature. Direct, human contact and interest in the farmer's individual herds and challenges are crucial. Veterinary advisers and cattle consultants could choose to see this as a profitable challenge, and local sector politicians should encourage and motivate to reach the goal throughout the process.

I would like to conclude this report with a list of suggestions for improvements to the current *Salmonella*-strategies in Denmark and suggestions for further research that can support the cattle industry and veterinary authorities in reaching the goal of eradication of *S.* Dublin by end of 2014:

- Risk-based surveillance methods might improve detection of newly infected herds and at the same time lower the cost of surveillance because active surveillance activities are aimed at high-risk areas or herds. E.g. it is possible that herds that have been in Level 1 for many years and are located in low-prevalence regions could be tested less frequently while herds with recent infection, infected neighbours, risky trade patterns etc. could be tested more frequently.
- Early-warning systems might aid in fast detection and reduce the duration of infection if management to block transmission routes are started quickly after an outbreak/new infection. A part of this can be to improve today's passive surveillance system. If it was more beneficial to report new outbreaks than today where it is a very undesirable situation this could improve early warning at low costs.
- Trace-back and trace-forth systems are used in Sweden that has obtained the situation we are aiming at with very few outbreaks per year. I recommend considering such methods as part of prevention of new outbreaks when we reach low prevalence.
- Improved slaughter procedures for cattle from infected herds when prevalence becomes lower, e.g. only slaughter of Level 2 herds on Fridays. This can improve food safety but also work as a motivator to intervene in the herds.
- Studies of human *S.* Dublin-cases to examine, if some of these cases could or should be avoided through improved biosecurity elsewhere in the farm-to-fork-chain than in the primary production.
- Studies to determine the prevalence of *S.* Typhimurium-infected cattle herds and whether there is a risk that this infection may increase in prevalence as S. Dublin decreases. Such phenomenon has been observed in other countries and it would be problematic to eradicate one type of *Salmonella* just to have to deal with another.

- Studies to evaluate of the effects on farm economics and animal health of *S.* Dublin infection and the eradication of the infection (this is currently being done as part of a PhD project at KU LIFE).
- Studies to evaluate socio-economic consequences of the eradication campaign should be estimated for the cattle sector and society.
- Studies to determine which import restrictions would help us reduce the number of human *S.* Dublin-cases and human *S.* Dublin-case fatalities from imported meat once after the infection is eradicated from Danish cattle.

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